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National Cancer Institute

NATIONAL CANCER INSTITUTE (U.S.) Division of Cancer Biology & Diagnosis

ANNUAL REPORT

October 1, 1984 through September 30, 1985

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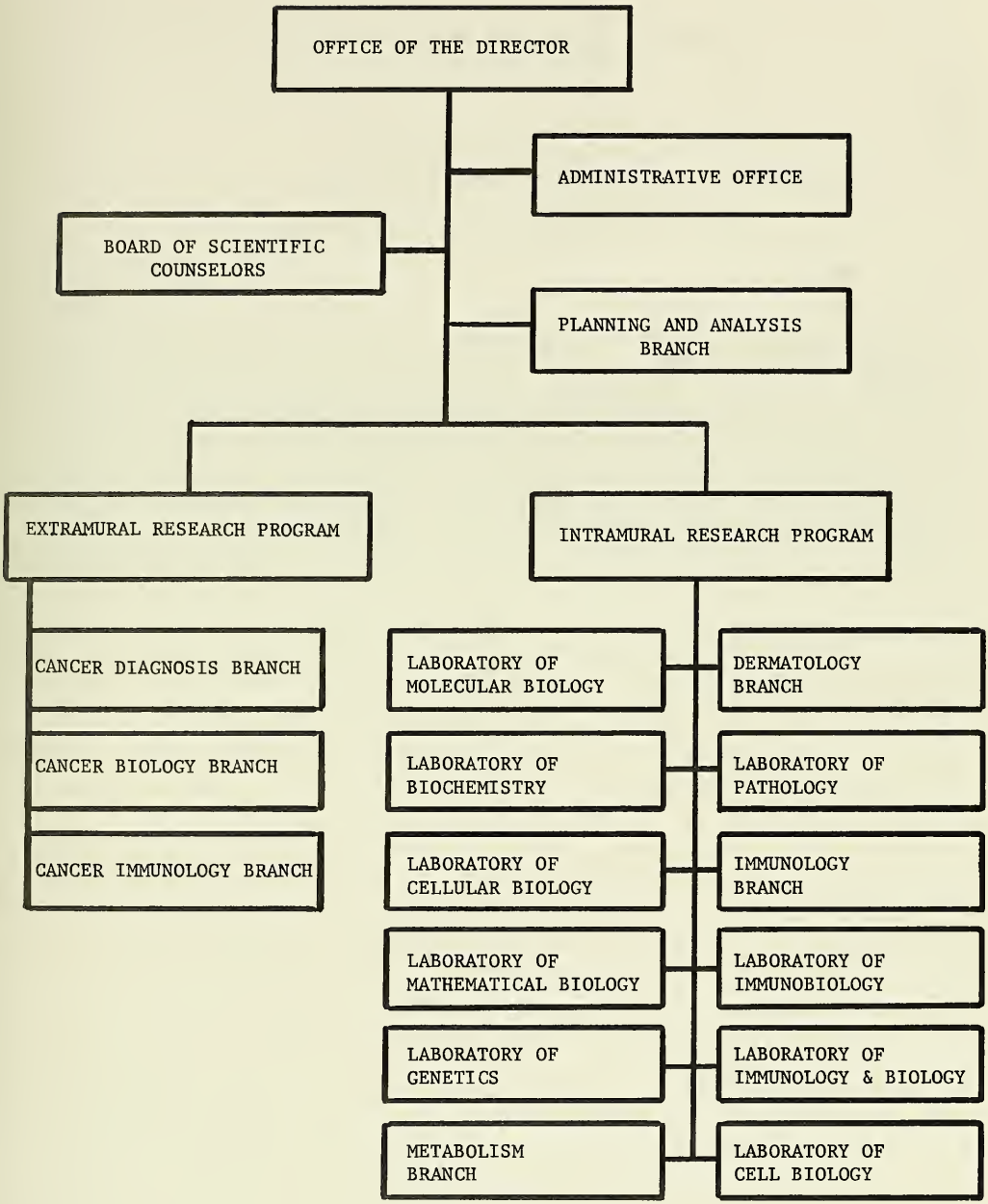
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DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
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INTRAMURAL RESEARCH PROGRAM

SUMMARY REPORT OF THE DIRECTOR

October 1, 1984 through September 30, 1985

INTRODUCTION

The Intramural Research Program of the Division of Cancer Biology and Diagnosis conducts laboratory and clinical research in cancer biology, cancer diagnosis, and immunology. A close coordination is maintained with other divisions of NCI in areas of common interest. This report will include descriptions of research programs of the intramural laboratories in the division. They are: the Laboratory of Genetics, the Laboratory of Mathematical Biology, the Laboratory of Biochemistry, the Laboratory of Cellular Oncology, the Laboratory of Molecular Biology, the Metabolism Branch, the Dermatology Branch, the Laboratory of Pathology, the Immunology Branch, the Laboratory of Immunobiology, the Laboratory of Cell Biology, and the Laboratory of Tumor Immunology and Biology.

Laboratory of Genetics

The Laboratory of Genetics (Dr. Michael Potter, Chief), conducts studies of how specific genes determine and regulate a variety of biological functions relevant to the problems of neoplastic development.

The induction of plasma cell tumors in BALB/c mice by the intraperitoneal injection of pristane or mineral oils provides a model system for studying the pathogenesis of a neoplasm. Recent studies have shown that abnormal proliferations of plasma cells in the pristane-induced oil granuloma can appear months before fully malignant transplantable tumors develop. Cells from these lesions are in an intermediate stage of neoplastic development and can be utilized to study the role of susceptibility-resistance genes, growth factors, mutagens, chromosomal translocations and other biologically active mediators in plasmacytoma development. A growth factor, PCT-GF, required by many plasmacytomas for growth in vitro, has been isolated and is being purified.

Significant progress has been made in understanding the structure and regulation of expression of two oncogenes, myc and myb, which are involved in plasmacytomas and lymphomas. These oncogenes, expressed in normal cells and tumors are overexpressed in certain inherited autoimmune diseases. Collaborative studies have established for the first time the orientation of the 3 mouse c-myc exons and the c-sis gene with respect to the centromere of chromosome 15 and corrected the published gene orientation of V_H, C_H and c-fos on chromosome 12. Further studies suggest that an important myc control sequence resides in close proximity to c-myc. Further studies on the regulation of myc expression have focused on two plasmacytomas which are superproducers of c-myc RNA (probably due to an increased rate of c-myc transcription and a diminished rate of myc mRNA breakdown). Similar studies of c-myb transcription are underway. In certain mouse myeloid tumors, helper virus integrates into the myb locus and causes premature termination of transcription and abnormally small myb mRNA. Efforts will be intensified to localize the site of rearrangements for both these oncogenes and to understand their effects on gene transcription.

Natural mouse populations have been used to study gene evolution in an attempt to define mutational mechanisms that operate on a variety of immunologically important genes. Recombinant DNA libraries have been constructed from six wild mouse species and have been screened with probes corresponding to the κ chain variable region family and the β chain of the T cell receptor. Homologous genes have been isolated from all species, and those from M. pahari have been sequenced. A comparison of genetic sequences between wild mouse species and inbred mouse strains suggest that each of the genes appear to be evolving in an independent manner and subject to different mutational processes. This finding is important to our understanding of the mechanisms involved in the introduction of polymorphisms in natural populations which subsequently effect corresponding phenotypes.

The FMR antigen found on most lymphomas induced by the Friend, Moloney, and Rauscher retroviruses has been studied as a model for understanding the antigenic structure of tumors and the origin of cell surface tumor-associated antigenicity. Considerable progress has been made in the characterization of the gp175 FMR antigen isolated from the Rauscher virus induced T-cell lymphoma RBL-5. The sensitivity and specificity of this assay may now make it possible

to develop DNA probes to identify the gene(s) that control gp175 and to determine the genetic basis for the origin of the antigenicity.

The primary structure of the monoclonal antibody (HyHEL-10) to hen egg white lysozyme (HEL) has been determined and a hypothetical model of this protein has been constructed. Using this scaled model, a complementary structure to the putative HEL-epitope has been found on HyHEL-10. The HyHEL-10 epitope is now being more precisely mapped by data obtained from heavy and light chain recombination studies. Considerable effort is being made to determine the three dimensional structure of a crystal of HyHEL-10 Fab combined with HEL. Work is proceeding on generating a system for producing and expressing further variants. These studies should provide a basis for designing antibodies with predetermined specificity.

Investigations of the mechanism by which murine leukemia viruses induce erythroid transformation have progressed. Comparisons of the molecular structure of two variants, $SFFV_P$ and $SFFV_A$, and formation of a recombinant virus have allowed localization of a region within the envelope gene that determines the ability of infected cells to differentiate in response to erythropoietin. Unlike Friend murine leukemia virus, the long terminal repeat (LTR) region of SFFV, does not carry sequences required for tissue-specific induction of leukemia. In addition, the generation of helper-free SFFVs has shown that helper virus is not required for in vitro transformation of erythroid cells, first stage acute erythroleukemia in mice or development in vivo of second stage tumorigenic cells.

Studies on the genetics of susceptibility to early erythroleukemia induced by Friend murine leukemia virus have led to the identification of a gene on chromosome 5, that plays a major role in resistance to this disease by preventing the replication of mink cell focus-inducing (MCF) viruses. This gene is either a structural gene or a regulatory gene for an MCF virus-related envelope glycoprotein that appears to block the cell surface receptor for MCF viruses. Additional genes, acting through unknown mechanisms, may also be involved in resistance.

Laboratory of Molecular Biology

The primary focus of research in the Laboratory of Molecular Biology (Dr. Ira Pastan, Chief), is the study of factors that control gene expression in animal cells and bacterial cells. In addition, there is a strong emphasis on investigating the role of the plasma membrane in receiving signals from hormones, growth factors, the extracellular matrix, and other cells and on determining how these signals are transmitted to the genetic apparatus. This information is being used to devise new ways of cancer diagnosis and treatment.

The results of previous studies on the pathway of receptor mediated endocytosis have been used to develop immunotoxins to treat human cancer. Research efforts have focused on Pseudomonas toxin (PE), which, when conjugated to antibodies, has very low toxicity on normal human cells. When PE is coupled to an antibody to the Interleukin 2 receptor (anti-Tac), the resulting PE-anti-Tac conjugate will kill cells from adult T-cell leukemia patients at 20 ng/ml. Comparable doses of PE anti-Tac have been administered to monkeys without significant toxicity. These studies indicate the PE-anti-Tac might be of use in treating adult

T-cell leukemia patients, and clinical trials are in progress. Pseudomonas toxin has also been coupled to antibodies that recognize antigens present on ovarian cancer cells. Some of these agents have very high activity in tissue culture and their efficacy is being explored.

Immunotoxins enter cells in endocytic vesicles termed receptosomes or endosomes, and must escape from this vesicular system into the cytosol in order to kill cells. Investigations are underway to explore methods of increasing the efficiency of penetration of immunotoxin molecules into the cytosol. Verapamil, a calcium channel blocker appears to enhance penetration, and verapamil analogs are being tested for their efficacy in vivo. An alternative approach is to lyse the membrane of endocytic vesicles. Previous studies showed that adenovirus enhanced the disruption of the membrane of the endocytic vesicle releasing its contents into the cytosol. Biochemical studies have now been carried out to determine the basis of this action. Recent studies suggest that the endocytic vesicle is acidic and when the virus reaches this environment, its proteins expose hydrophobic residues that penetrate the membrane of the vesicle. The vesicle appears to be under osmotic tension and this high osmotic pressure probably causes rupture of the vesicle at the point of adenovirus insertion.

Another series of experiments have focused on providing a better understanding of the genetic and biochemical basis of multiple drug-resistance in chemotherapy-resistant human tumors. A human epitheloid cancer cell line (KB) has been mutagenized and used to select clones displaying high level multi-drug resistance. Highly resistant cell lines contain amplified genes which code for the drug resistance. A revertant cell line which has lost multiple drug resistance has lost the amplified sequences. A DNA probe has been obtained which recognizes a messenger RNA (overproduced in multi-drug resistant cell lines) which probably codes for a protein having a role in multi-drug resistance. This DNA probe is currently being used to determine if the findings in cell culture are relevant to human cancers.

Internalized ligands are delivered into the cell together in the same vesicles, but may then separate to either recycle to the cell surface or be delivered to lysosomes. Their segregation occurs in an elaborate, but ill-defined, array of membranous elements of the "trans" domain of the cell, that is connected functionally with the trans face of the Golgi stacks, as well as the plasma membrane. Studies are underway to define morphologically the elements of this "trans" domain of the cell using specific morphologic cytochemical markers. These studies should help to define the organelle locations in which segregation of proteins and ligands occur and should enable a better understanding of the underlying biochemical mechanisms that might be responsible for these events.

Previous morphologic studies have suggested that the coated pits of the Golgi region are important in the intracellular sorting of ligands entering the cell by endocytic vesicles. Using a new biochemical procedure, the transit of epidermal growth factor (EGF) was sequentially followed. It was found that EGF drives the EGF receptor first through coated pits at the surface and later through coated pits of the Golgi on the way to the lysosomes. In similar studies it was shown that the transferrin receptor, which is not directed to lysosomes, is only found in the first plasma membrane associated coated compartment. These studies support the conclusion that the clathrin coated membranes of the Golgi have an important role in directing the EGF receptor (and

probably other receptors) to lysosomes by concentrating them out of the Golgi compartment.

Progress has been made in genetic analysis of cultured somatic cells to determine the role of cAMP in regulation of cell growth and morphology. Of particular interest is the involvement of tubulin in spindle formation and the mechanism whereby human cancer cells develop resistance to multiple drugs. Dominant mutations conferring resistance to cAMP-mediated growth inhibition have been transferred by DNA-mediated gene transfer to sensitive recipient cells. This assay is being used to clone the gene encoding a mutant regulatory subunit of type I cAMP dependent protein kinase. A mutant tubulin gene conferring colcemid-dependence and a specific spindle defect has been transferred and amplified in recipient cells. Several independent multi-drug resistant human KB carcinoma cell lines, which express specific amplified DNA sequences have been developed.

The role of ADP-ribosylation of nuclear proteins in mediating the genetic regulation effects of steroid hormones has been examined.

The major excreted protein (MEP) of transformed mouse fibroblasts, a novel and activatable lysosomal protease, has been found to be regulated at the level of transcription by the tumor promoter, TPA, and growth factor, PDGF.

Work has continued on the analysis of the functions of adhesive glycoproteins and their receptors. Structural comparisons between species indicated that the two major forms of fibronectin are derived from a single gene. An evolutionarily conserved region was shown to be a critical, specific recognition signal for fibronectin-mediated cell adhesion and movement in vitro, and recent experiments have shown that such fibronectin peptides inhibited two major migratory processes in embryonic development.

The binding of fibronectin to cells occurs via a moderate-affinity receptor. The putative receptor for fibronectin was isolated and characterized. Collagen receptor function was shown to modulate fibronectin receptor function, and studies are in progress to characterize the mechanisms of action of the putative fibronectin and collagen receptors, and to test the efficacy of synthetic peptides in inhibiting a variety of adhesive and migratory events, including platelet function and tumor cell invasion.

Fibroblasts which have undergone malignant transformation show an important transcriptional inhibition of the type I collagen genes and other transformation sensitive genes. To understand the mechanisms which cause this inhibition a series of mutant cell lines were developed in which the expression of these genes is restored. The mutants are pleiotropic and contain a mutation which either directly or indirectly controls the expression of the type I collagen and fibronectin genes. Isolation of the mutated gene(s) is in progress. These studies should help dissect the pathways of cellular reactions which are triggered by the oncogenic proteins and which cause the alterations in growth control and other aspects of the transformed phenotype of these cells.

Studies have continued on the regulation of *gal* operon, *rho* gene and the molecular basis of cyclic AMP action in gene transcription in *E. coli*. The *gal* repressor binds to the two operators of the *gal* operon and plays a dynamic role in modulating transcription. Instead of repressor sterically blocking RNA

polyermase entry to promoter, two molecules of repressor bound to the two operators change the conformation of the promoter DNA structure making it inadequate for transcription initiation.

Cyclic AMP complexes with its receptor protein, CRP and the cyclic AMP. CRP complex binds in a sequence specific way to DNA to modulate gene expression. By isolating and studying mutants of CRP, which modulate the gene without cyclic AMP, it has been possible to precisely define the amino acids which interact specifically to cause the allosteric shift. Genetic identification and cloning of three genes responsible for regulation of capsular polysaccharide synthesis suggest that one of the regulatory proteins may be sensitive to proteolysis by the Lon proteolysis system. Studies are in progress to determine the in vitro specificity of Lon proteolysis.

Laboratory of Biochemistry

The research program of the Laboratory of Biochemistry (Dr. Maxine Singer, Chief), includes studies on eukaryotic development and differentiation, the mechanism of DNA replication, the biosynthesis of macromolecules, the regulation of cellular processes at the level of gene expression and macromolecular interactions, the organization of eukaryotic genomes and the biology of T-cells.

The objective of one research project is to determine the mechanism by which vitamin C (ascorbic acid) controls connective tissue metabolism. Previous studies showed that decreased synthesis of both collagen in bone and proteoglycan in the cartilage of scorbutic guinea pigs was directly related to the extent of weight loss rather than to defective proline hydroxylation. These and other results suggest that ascorbate deficiency indirectly produces these effects by inducing a chronic fasting state, and that decreased collagen production in both bone and cartilage of acutely fasted animals is not due to an increase in degradation but to decreased synthesis caused by a reduction in the levels of procollagen mRNA.

Three proteins, α-polymerase, β-polymerase and the single-strand nucleic acid binding protein termed helix destabilizing protein are being studied in an ongoing investigation of the mechanism of mammalian DNA replication. Six cDNAs for the binding protein were isolated and sequenced. A cDNA clone for rat β-polymerase was sequenced. This DNA appears to be a full-length mRNA copy encoding the 40KDa enzyme. Similar results were obtained with human DNA and the gene was localized to human chromosome 8. β-polymerase levels were found to be significantly elevated in lung cancer cells and in cells from patients with ataxia telangectasia. One cDNA clone for rat α-polymerase was also obtained and is being further characterized.

Continued studies are in progress on Intracisternal A-particle (IAP) genes, a family of retrovirus-like genetic elements endogenous to mice and other rodent species. Recent data linked IAP genes to a class of T-cell immunoregulatory factors that modify IgE production in differentiating B-lymphocytes. Current investigations are seeking to determine whether analogous regulatory factors for other types of immunoglobulins are also IAP-related. Another series of experiments are directed towards determining the possible role of IAP antigen expression in the pathogenesis of a genetically determined insulin-dependent diabetes in mice.

Additional efforts have focused on a subset of mouse IAP genes. These type II IAP genes show both deletions of various sizes, rearrangements, and insertion of a particular 300 base pair sequence element. This suggests that they were formed through multiple recombinational events. The inserted sequence element has not been detected outside of Mus musculus and some closely related mouse species.

The development of methods for gene transfer to mammalian cells has continued with a goal of using these techniques for gene mapping, analysis of gene expression, and cloning eukaryotic genes. Analysis of DNA isolated from a large group of independent somatic cell hybrid lines that segregate human chromosomes has enabled localization of human genes to specific chromosomes with particular emphasis on genes potentially involved in human neoplasia. These include known proto-oncogenes, translocation breakpoints in human tumors, and genes involved in DNA replication or activation of carcinogens.

A GL-13 BC guinea pig leukemia model has been developed that appears to have many similarities to human chronic myelogenous leukemia (CML). Identification of the transforming genes in these leukemias is required to firmly establish this relationship. However, Southern analysis of guinea pig leukemic DNA did not detect any rearrangements of c-abl or several other cellular proto-oncogenes. Efforts to detect alterations in the guinea pig c-abl transcript, similar to those consistently reported in human CML, are in progress.

The metallothioneins (MT) are being analyzed by molecular genetic and biochemical approaches as a model for the regulation and function of eukaryotic genes. Human MTs are encoded by a complex multigene family that includes at least five functional members clustered on chromosome 16. Two highly homologous human MT-I isoform genes have been cloned and sequenced, permitting analysis of cell type specific expression. Activated ras oncogenes in cells were also found specifically to enhance MT gene transcription and the molecular mechanism for this phenomenon is being investigated. The yeast Saccharomyces cerevisiae is being used as a model system for studying the structural basis for the detoxification and autoregulatory functions of the MT-like protein copperthionein.

Understanding the mechanisms that achieve gene regulation provides a basis for understanding eukaryotic development and differentiation. Unit copy bacterial plasmids that are particularly amenable to detailed genetic analysis have been chosen for study. Fertility factor F and prophage P1 are composite replicons that are similarly organized at a plasmid maintenance region, although essentially nonhomologous in their DNA sequences. Particular isolated basic replicons from F and P1 that are similarly capable of dnaAts integrative suppression exhibit a single region of DNA homology that corresponds to a tandem pair of DnaA protein binding sites. Recent studies have shown that some replicons that are capable of dnaAts integrative suppression and that bear DnaA protein binding sites can replicate in the total absence of dnaA function, whereas others cannot. The different kinds of replicons that are present in F and P1 may serve to enlarge the range of bacterial hosts and environmental circumstances in which these plasmids replicate.

Gene regulation is being studied by probing the structure of DNA associated with proteins in chromatin. The sequential arrangement of nucleosomes along the chromatin fiber is punctuated by highly nuclease-sensitive sites. These preferentially accessible sites may function as points of entry to the DNA for

RNA polymerase and control proteins. Using a new exonuclease protection for mapping protein binding sites in chromatin, two such sites for both the hsp 82 and hsp 70 genes were found. This suggests that heat shock genes are activated by the sequential binding of at least two protein factors. New assay procedures are being used to purify the two proteins.

Using cloned cDNA probes, the genomic sequences have been isolated and partially or completely obtained for the following proteins: alpha skeletal actin, alpha cardiac actin, beta cytoplasmic actin, myosin light chains 1-3, vimentin, pyruvate kinase, glyceraldehyde phosphate dehydrogenase, and a nerve growth factor induced gene. The 5' and 3' probes specific for the various chicken actin genes have been used to measure levels of expression and to monitor the pattern of regulation of the actin genes in these various mouse cell backgrounds. The entire sequence of the alpha cardiac actin gene was determined and compared to the alpha skeletal actin gene. Even though both genes are expressed in developing muscle there is no evidence that there is a common regulatory mechanism controlling tissue specific expression or developmental regulation.

Recombinant DNA technology is being used to study the regulation of expression of the immunoglobulin gene family. Previously, it had been shown that the complete kappa gene is transcribed after transfection into myeloma cells but not in non-lymphoid 3T3 or L cells. Thus, the lymphoid cells appropriately regulate the kappa gene even when it is not in its usual chromosomal environment. A 200 base pair region of DNA downstream of the promoter is necessary for gene transcription in myeloma cells and is a cell-specific enhancer. Recent data shows that the promoter for an immunoglobulin kappa light chain gene is also strongly specific for lymphoid cells. Thus, both regulatory elements participate in cell specific gene expression.

Additional studies have been directed towards understanding the role of the interaction of cytoplasmic myosins with actin filaments in the various motile activities of the cell. Whereas both smooth muscle and cytoplasmic myosins are similarly regulated, their mechanism of activation is different.

Calmodulin is a ubiquitous calcium-binding protein which recognizes changes in the intracellular concentration of calcium induced by external stimuli and transmits this information to cellular proteins whose activity is thereby altered. The mechanism of regulation of cellular processes by calcium and calmodulin is a topic of continuing research interest. Previous work showed that calcium binding to calmodulin is an ordered stepwise process, which generates at least three different conformers. Recent efforts have been devoted to study of the interaction of calmodulin with several calmodulin-regulated enzymes. The data confirm that this interaction requires the integrity of different portions of the calmodulin molecule and that the enzymes have different requirements for activation by or interaction with calmodulin. The isolation and characterization of the interaction sites of calmodulin with calcineurin are underway. This enzyme may play a major role in the calcium regulation of protein phosphorylation and thereby be a coupling factor between calcium and cAMP.

Additional studies focus on two different classes of sequences that are highly repeated in primate genomes: the LINE-1 family (whose members are interspersed in the genome) and centromeric satellite DNA (which appears in long tandem arrays at centromeres). The African green monkey genome and human DNA have

been selected for study. The major primate family of highly repeated long interspersed DNA sequences (LINE-1) includes several thousand 6 kbp long units that terminate in an A-rich stretch. In addition there are more than 10^4 dispersed copies of truncated, rearranged and deleted LINE-1 segments in primate genomes. Recent data suggests that the LINE-1 family includes one or more functional genes and that these are expressed in cleavage stage embryos.

Earlier work indicated that the organization of one monkey centromeric satellite DNA, deca-satellite, is highly polymorphic in individual members of the species and that the amounts of deca-satellite and α-satellite, vary (independently) in individual genomes. In an effort to understand the maintenance of such extensive but variable DNA sequences, analysis of junctions between satellite and unique genomic sequence has been initiated. Several observations suggest that these cloned "junction" segments contain recombinogenic segments.

Continuing efforts have been made to develop methods for the specific isolation of immune cells in order to study their roles in cellular immune reactions. The subsets of $Lyt2^+T$ cells that are distinguished by the rat monoclonal antibody "B4B2" have been studied. Both $B4B2^+$ and $B4B2^-$ lymphocytes were active in the in vivo graft vs. host reaction. In another study, it was shown that in vitro purified immune $Lyt2^+T$ cells had in vivo antitumor activity.

Since many tumors fail to elicit an effective immune response, "xenogenization" (induced mutation in tumor cells to increase their immunogenicity) is a potential approach to cancer immunotherapy. In a study of the immune response to such cells, a humoral response was obtained to a chemically xenogenized murine tumor cell line that was previously only capable of eliciting a cellular immune response.

Myeloblast maturation factor (MMF) has been isolated from guinea pig serum in a highly purified form. Pure transferrin isolated from human serum demonstrated potent MMF activity. In studies aimed at the identification of molecular mechanisms responsible for arrested myeloblast maturation in the blast crisis stage chronic myelogenous leukemia of (CML), it was found that leukemic myeloblasts from that stage of the guinea pig CML model failed to respond to MMF under conditions in which this factor induces maturation in normal myeloblasts. Testing for defective receptors is planned, and efforts to identify the genes in CML myelocytes that are responsible for the defective maturation program in these cells are continuing. Using high performance liquid chromatography (HPLC), it is possible to detect defects in the protein phenotypes of mature granulocytes from CML patients. Present results indicate that a quantitative decrease in the height of one or more protein peaks in the HPLC pattern can be used to predict the onset of the accelerated stage several months in advance of other clinical tests.

Technical improvements continue to be made in the synthesis, purification, and fractionation of the carboxymethyl-dextrans (CMDs) used in the ion-exchange displacement (IED) chromatography of proteins. This method can be used to completely separate two proteins that differ in isoelectric point only by 0.1 pH unit. This remarkable capacity is of great importance to the use of HPLC columns for preparative scale purifications in both industry and the laboratory.

Laboratory of Mathematical Biology

The research activities of the Laboratory of Mathematical Biology (Dr. Jacob Maizel, Chief), include studies of macromolecular structure and function, membrane structure and function, immunology, pharmacokinetics, and computational and modeling methodology. Application of theoretical understanding to the biological systems which serve as models for aspects of the cancer process is accomplished through the use of advanced computing.

Computerized analyses are used extensively along with techniques of biochemistry, virology, and electron microscopy to study various virus-cell systems. Sequences of picornaviruses, (such as polio, rhino, hepatitis, and foot-and-mouth disease), are examined for relationships within the family and other known and hypothetical proteins. Secondary structures of the RNAs have been found to vary with respect to pathological and sequence variances. Studies of adenoviruses are providing information on early events in virus replication in which the cell's metabolism is subverted to viral functions, and on late events during which assembly and morphogenesis occurs.

New analyses of proteins and nucleic acids are being developed. Graphic representatives revealing homology, and reverse complementarity are coupled with numerical methods to aid the prediction of secondary structure. Protein secondary structure is predicted from amino acid sequences. New sequences are being compared with computerized databases to detect relationships with known proteins. Planning and procurement is underway for the development of an advanced supercomputing facility. This machine will be the first scientific computer dedicated entirely to biomedical research.

Studies of biological macromolecules and their properties have continued. Both long range and short range interactions in proteins have been investigated. Effective interaction energies between all types of pairs of amino acids have been obtained. These energies are being applied to locate hydrophobic nuclei in an attempt to incorporate them into a protein folding scheme. Construction of molecular models has been expanded to include membrane channels composed of amphipathic helices, DNA local conformations and DNA-protein interactions. Computer color graphics systems assist in these modeling efforts. The effects of drugs and carcinogens on the electronic structure of DNA are calculated.

General purpose computer programs (SAAM, CONSAM) for the simulation of compartmental models of bio-kinetic systems continue to be developed for use by investigators not sophisticated in mathematics or computer services. The programs which make up SAAM have been revised so that they will execute on IBM 43XX series of computer. In addition it has been tested on a new series of dec computers, MicroVAX I. This is the first microcomputer capable of executing all the programs which make up SAAM. The SAAM programs have been used to study the metabolism of chylomicrons in rats. Using this model it was determined that the immediate nutritional status of the rat has a major effect on the mechanism of triglyceride metabolism. Recent studies suggest that hydrolysis of triglycerides will be decreased in the low fat nutritional state recently associated with cancer prevention.

A continuing research interest is the study of membrane structure and function. Research projects focus on the insertion and organization of molecules (proteins, lipids) in membranes, interactions of membranes with toxins, viruses,

channel proteins and polypeptides, and membrane fusion. Spectroscopic techniques are used to study lipid-protein interactions and membrane fusion, and conductance across planar black lipid membranes is measured to approach questions of membrane destabilization and channel formation.

Studies on membrane fusion focus on reconstitution of viral spike glycoproteins into lipid vesicles and on membrane fusion mediated by the resulting virosomes. A theoretical framework is being developed to model aggregation and fusion kinetics. Efforts are directed at constructing virosomes which fuse with cells in order to deliver materials to the target cell membrane or cytoplasm.

Studies on channel-formation with cytolysin isolated from large granular lymphocyte tumors and from cytotoxic T lymphocytes are being pursued with lipid vesicles and planar bilayers to examine the mechanism of its lytic action. Recent experiments suggest that Interleukin-2 (IL-2) might form channels in bilayers, and this might have important implications for the biological action of IL-2. The interaction of adenovirus with lipid membranes has been studied in an attempt to better understand the mechanism by which adenovirus enters the cytoplasm of a host cell.

Work has continued on evaluating the effectiveness of delivering monoclonal antibodies via subcutaneous injection to the lymphatic system, as a method of improved diagnosis and therapy of lymph node metastases. Antibody-toxin conjugates and antibody-alpha emitter conjugates have been evaluated for their possible usefulness in the treatment of tumor in lymph node and for modulation of the immune response to tumors. Animal studies have been extended directly into clinical protocols for the detection of melanoma, lymphoma, breast carcinoma, and non-small cell lung carcinoma in lymph nodes. Results with lymphoma have produced the most efficient imaging of tumor cells yet achieved in humans by any non-invasive technique.

For tumor cells far from the nearest lymphatic or blood vessel, binding of an antibody (or other ligand) may be limited by the rate at which the molecules can "percolate" through the extracellular space. Spatial and temporal profiles of immunoglobulin distribution generated by diffusion and convection through tumors have been analyzed. Of particular interest is the finding that low affinity antibodies are sometimes more effective in therapy than higher affinity ones.

Theoretical studies in immunology focus primarily on the development of new techniques for predicting the tertiary structure of immunoglobulin family molecules with applications to cell interactions; prediction of sites recognized by antibodies and T cell receptors and the development of improved vaccines based on such predictions; the development and application of secondary structure predictive methods, the development of physical chemical principles governing macromolecular stability and development and application of pattern recognition algorithms to identify various sites on nucleic acids (such as exon/intron boundaries).

Collaborative research efforts continue to explore the area of B-DNA distortions. Feature enhancement and feature detection techniques have been developed to enable the user in an interactive environment to discover new patterns of potential interest. These techniques have indicated the existence of morphologic structures which appear to be correlated to functionality within the molecule. Work has also begun on a new algorithm to measure similarity among

secondary structures of RNA molecules. This technique should permit the determination of multiple levels of similarity among several molecules of the same or different classes.

Laboratory of Cellular Oncology

The Laboratory of Cellular Oncology (Dr. Douglas Lowy, Chief), conducts research on the cellular and molecular basis of neoplasia. Tissue culture cell systems and animal models are used to study the induction and maintenance of benign and malignant neoplasia and reversal of the neoplastic state, to elucidate structure-function correlations through detailed examination of individual genes which have been implicated in neoplasia, and to examine spontaneous tumors for the presence of exogenous genes or altered cellular genes.

Laboratory investigations continue to focus on the mechanisms by which tumor viruses or cellular genes contribute to oncogenesis and to devise approaches to prevent or reverse such changes in cells. Various ras oncogenes and the ras containing Harvey murine sarcoma virus (Ha-MuSV) have been chosen for study. The ras oncogene has been used to show that an activated oncogene may induce metastatic potential in some cells, but not in others. Introduction of Ha-MuSV DNA into a human breast cancer cell line renders these cells tumorigenic for nude mice that have not received estrogen, although the parental cell line requires exogenous estrogen to be tumorigenic. Thus, onc gene activation may be one mechanism by which estrogen independence occurs clinically. Site directed mutagenesis has identified a cysteine residue near the C-terminus of all ras genes that is absolutely required for the transforming function of the protein, its membrane localization, and its binding of lipid. Using frame shift and deletion mutagenesis, two genes in Bovine papillomavirus have been identified that can independently transform established mouse tissue culture cells. The protein product of one of these transforming genes has also been identified and is being characterized further. This gene may be involved in human and animal tumors associated with papillomaviruses. Detection of the homologous protein in human papillomavirus infections may prove to be useful diagnostically.

Another project is designed to investigate the roles of cellular oncogenes and retroviruses in neoplasia and to use viral mutants to study the regulatory mechanisms of gene expression associated with cell differentiation and oncogenesis. Oncogene amplification, rearrangement, and expression have been studied in several different malignant tumor cell lines. In a model system that employed an anchorage dependent mink cell line non-productively infected with Moloney sarcoma virus (which contains the v-mos oncogene), superinfection with a novel dualtropic mouse retrovirus induced anchorage independent cell growth that correlated with a marked amplification and increased expression of the v-mos oncogene.

Continuing progress has been made to elucidate the molecular genetics of neoplastic transformation of normal tissues and the purification and function of biological modifiers that may be important to host defense. A transforming gene detected in a human hepatocarcinoma cell, was shown to be significantly enhanced by treatment with aflatoxin (AF) B-1-epoxide, a potent carcinogen. A 3.1 kb DNA fragment has been molecularly cloned and is being sequenced. Preliminary data suggest that the fragment contains sequences related to retroviral regulatory elements. Further studies of this clone and its interaction with

AFB-1-epoxide may yield significant insights into carcinogenesis. In biological modifier studies, a lymphokine called cytotoxic cell differentiation factor (CCDF) has been studied functionally and purified. CCDF is required, in conjunction with IL-2, to form lymphokine-induced cytotoxic cells (LICC), which may be an important host defense mechanism against neoplastic cells. Based on their cell surface markers, the LICC induced by these factors represent a unique class of cytotoxic cells. The extent of involvement of this lymphokine system in surveillance against neoplastic cell and the in vivo anti-tumor activity of these LICC are currently under study.

Methylcholanthrene induced BALB/c sarcoma (Meth A) which can be passed both in vivo and in vitro, is being used as a model system to study the mechanism by which interferon (IFN) acts on cells. In this system, comparison of the response in cell culture with that in the intact mouse makes it possible to analyze the direct action of IFN on Meth A cells separately from the host defenses. These experiments confirm the necessity of functional T cells in order for IFN to exert its antitumor effect. The results obtained suggest that the major effect of IFN on chemically induced sarcomas is mediated through the host immune response, rather than by its anti-cellular activities.

Studies of several groups of murine leukemia viruses and their relationship to many inbred strains of mice have contributed significantly to our understanding of many viral diseases. Using a Mus dunni cell line, several new viruses have been isolated. Biological and biochemical characterization of some of these new viruses and their role in pathogenicity may prove useful to our understanding of disease production.

Laboratory of Pathology

The Laboratory of Pathology (Dr. Lance Liotta, Chief), is responsible for all the diagnostic services in anatomic pathology for the Clinical Center of the NIH. About 5,500 surgical specimens or biopsies were accessioned in the past year, and approximately 1,000 specimens of fresh human tissues, were furnished to NIH scientists in various laboratories. Clinicopathological studies in pediatric neoplasms, endocrine tumors, soft tissue sarcomas, acquired deficiency syndromes, and breast cancer are in progress.

The cytopathology section provides diagnostic services in cytology, diagnosis, hematopathology, and electronmicroscopy. A new immunoperoxidase method employing a battery of antibodies to T and B cell markers has been developed which can distinguish malignant lymphoma cells from reactive lymphoid cells.

Recent studies have demonstrated that a unique, previously uncharacterized tumor of childhood, peripheral neuroepithelioma is distinct from childhood neuroblastoma, in that it lacks N-myc expression, and expresses c-myc. Unlike neuroblastoma, it never produces catecholamines, instead contains purely cholinergic neurotransmitter enzymes. Clinical studies based on these results are underway.

Another area of active investigation is childhood rhabdomyosarcoma. Tumor material has been characterized by a variety of techniques, and has demonstrated two basic types of rhabdomyosarcoma which on retrospective analysis have shown a strong prognostic correlation. An international review panel has been convened to develop morphologic criteria for categorization of childhood

rhabdomysarcoma into favorable and unfavorable histology. This will be the first time such uniformity of criteria for treatment purposes will have been achieved in pediatric oncology.

It was shown that the type of matrix molecules produced by pediatric "round cell" tumors can be a clue to their cell of origin. A previously undescribed high molecular weight matrix protein has been identified and characterized. Neurone-specific enolase was found to be a reliable marker for neuroblastoma and to aid in the differential diagnosis of neuroblastoma versus other solid tumors of childhood.

Research on immunochemistry of complex carbohydrates has focused on the determination of carbohydrate structures of glycoproteins, the development of hybridoma antibodies against oligosaccharide haptens, and immunochemical studies of cell surface glycoproteins.

Carbohydrate chains released by trifluoroacetolysis of whole tissues, tissue fractions, or cells grown in culture, are easily recovered in nearly quantitative yield and reconstituted to their native form. New methods have been developed for the analysis of oligosaccharides. It is anticipated that the repertoire of oligosaccharide chains produced by cells or tissues will reflect states of cellular differentiation and reveal potential cell surface markers. Membrane glycoproteins behave as carbohydrate or peptide antigens and occasionally express antigens that combine specific structural elements from both sugar and peptide moieties. Studies are in progress to characterize the immune responsiveness to cell surface glycoproteins in a systematic way.

The mouse immune response to streptococcal group A carbohydrate (GAC) utilizes many different gene segments from a diverse array of precursor variable heavy chain (Vh) genes. The response is clonally restricted in each mouse immunized, but each mouse appears to express a different clonally homogeneous antibody in its serum in spite of genetically common background. Each of these antibodies contains highly homologous Vh gene segments, and identical Dh, and Jh segments. The serum response to GAC is entirely deficient in CBA/N mice which makes this an ideal system in which to investigate possible constraints on gene segment utilization.

In order to further the study the interaction of tumor cells with laminin, a glycoprotein of basement membranes, the domain of the laminin molecule which attaches to the tumor cell and the specific laminin receptor have been identified. Molecular clones of the laminin receptor have been isolated and characterized. A fragment of the laminin molecule which binds to the receptor and blocks attachment will inhibit metastases in animal models. Laminin receptors can be readily measured in human breast carcinomas. Attempts to correlate receptor content with clinical stage of disease have demonstrated a higher content of exposed laminin receptors in breast cancer cases with two or more lymph nodes positive for metastases compared to cases with no metastases. Clinical trials are being set up to study the localization of metastases by labeled laminin fragments.

Type IV collagenase is an important basement membrane degrading metalloproteinase that malignant cells produce and secrete to facilitate their traversal through blood vessel walls as well as during transition from in situ to invasive carcinoma. Human type IV collagenase was isolated from culture supernatants of metastatic melanoma cells, and is being characterized.

Malignant tumor cells must be highly motile while invading tissue and metastasizing to distant sites. A number of metastatic cell lines produce and respond to autocrine motility factors. A partially purified material from the conditioned media of a human melanoma cell line was found which induces a strong chemotactic response in the producer cells. It appears to exert its action by perturbing membrane phospholipid metabolism of the cell. In studies of 3T3 cells and their transformed metastatic counterparts, it was observed that the transformed cells produce and respond well to autocrine factors, while the nontransformed cells do not. These results suggest than an important characteristic of malignant cells is their ability to produce and respond to autocrine motility factors.

A variety of techniques are being used to identify specific genetic elements whose expression is altered in metastatic cells. To date, several molecular clones which code for mRNAs which are expressed to a differential degree in metastatic versus nonmetastatic lines have been isolated. Levels of mRNA for the major excreted protein (MEP) of transformed murine cells are increased in the nonmetastatic cells, whereas levels of mRNA for type IV collagen are increased in the metastatic cells.

Studies with NIH 3T3 cells as well as diploid fibroblasts have confirmed the ability of $\underline{ras}^{H}$ genes to induce metastatic potential as well as transformation and tumorigenicity. However, transformation itself or the ability to grow as a tumor was shown to be insufficient to result in metastasis. The normal cellular counterpart of the $\underline{ras}^{H}$ oncogene can also transform NIH-3T3 cells under certain conditions. However, NIH-3T3 cells transformed by this construction, while highly tumorigenic, do not metastasize. The induction of metastatic potential in those cells is being attempted in gene transfer experiments.

An active research program is carried out on the immunological characterization of malignant lymphomas. All patients with newly diagnosed lymphomas or recurrences are studied for phenotypic and functional markers. This information is utilized to study the relationship of malignant lymphomas to the normal immune system, to develop improved classifications of disease, and to distinguish new clinicopathologic entities.

A recent laboratory study demonstrated lymphokine-induced phagocytes in the angiocentric immunoproliferative lesions (AIL). Phagocytosis-inducing (PIF) activity was demonstrated in patients with AIL, but not other peripheral T-cell neoplasms, B-cell lymphomas, or Hodgkin's disease, and this PIF activity was comparable in both the "benign" and "malignant" phases of their disease. This suggests that AIL represents a single nosologic entity, and the "conversion" to lymphoma does not occur.

A functional study of follicular non-Hodgkin's lymphomas showed that the neoplastic cells could be induced to secrete monoclonal immunoglobulin in vitro only when allogeneic helper T lymphocytes were added to the culture. T cells derived from involved lymph nodes were not capable of mediating such help, and appeared to exert a suppressive effect. Another study demonstrated that in some patients with follicular lymphoma, mature non-neoplastic T cells were numerically predominant and appeared to represent a positive host immune response. These studies suggest that the neoplastic cells of follicular lymphoma are susceptible to host immunoregulation.

A new research program has been initiated to study the molecular basis of the diagnosis of human lymphoproliferative disease. The T- and B-cell lineage of diffuse, aggressive lymphomas as determined by phenotypic studies can be confirmed by detection of appropriately rearranged T-cell receptor or immunoglobulin genes. In some patients' tissues which lack histologic evidence of neoplasia, malignant lymphoma can be identified only by these techniques. Molecular genetic analysis has also shown that a subclass of diffuse, aggressive lymphomas contain the t14,18 chromosomal translocation characteristic of follicular lymphoma. This suggests that many of these high grade B-cell lymphomas may originate as follicular lymphomas both clinically and at the molecular level.

Precursor T-cell neoplasms occupy sequential differentiation compartments during early T-cell development. Monoclonality and T-cell lineage were demonstrated by rearrangement of the T-cell receptor in Tγ lymphoproliferative disorder. Angio-immunoblastic lymphadenopathy, which frequently progresses to malignant lymphoma, contains monoclonal rearrangements of either immunoglobulin genes, T-cell receptor genes, or both simultaneously.

Although in most patients with malignant lymphoma, sequential studies show clonal fidelity in follicular B-cell lymphomas there is a high frequency of clonal evolution. Such clonal evolution allows for the escape of the malignant cells from anti-idiotypic antibody. Possible mechanisms of clonal evolution include somatic mutation, new variable gene rearrangements, and heavy chain constant region isotype switching. These observations have important significance for treatment strategies for the follicular lymphomas which, although clinically low-grade, have been refractory to cure with current regimens. The capacity of follicular lymphomas to frequently alter their surface membrane idiotype may allow them to escape detection by the host immune response and this may account for the clinically observed waxing and waning clinical course of disease.

An active research interest continues in the regulation of cell growth by transferrin receptors. Normal cells regulate transferrin receptor appearance by controlled tissue-specific growth factors and their receptors. However, in malignant cells this regulation is lost and transferrin receptor expression becomes constitutive. Both growth factor dependent and constitutive transferrin receptor expression are calcium dependent while transcription of the IL2 receptor gene and genes for c-myc and c-myb are not.

Dermatology Branch

The Dermatology Branch (Dr. Stephen Katz, Chief), conducts both clinical and laboratory research studying the etiology, diagnosis and treatment of inflammatory and malignant diseases involving the skin and the host's response to these diseases. The Branch also provides dermatology consultant services to all other groups in the Clinical Center.

Studies of the immunopathology of skin diseases have continued, with the main emphasis on the effects of ultraviolet radiation (UV) on epidermal immune responses. Ultraviolet light irradiation modulates the antigen presenting function of Langerhans cells such that UV irradiated epidermal cells stimulate allogeneic T cells poorly if used immediately after irradiation and stimulate vigorously if taken 3 days after irradiation. After UV, an $OKT6^{-}DR^{+}$ melano-

phagic cell responsible for enhanced stimulation appears in the epidermis. This cell has been characterized further. In additional studies, it was found that UV inhibits the ability of Langerhans cells and various other antigen presenting cells to process various types of antigens. This inhibition may be responsible for the ultimate generation of suppressor cells.

A new research program has been established to study autoantibody-mediated skin diseases in order to further an understanding of the pathophysiology of these diseases as well as the structure and function of normal epidermis and epidermal basement membrane zone (BMZ). Antibodies in these diseases define molecules in the normal epidermis. Antigens defined by bullous pemphigoid (BP), pemphigus vulgaris (PV), and pemphigus foliaceus (PF) have been characterized and the cells that synthesize these antigens have been defined. It is now possible to use specific antibody binding to make diagnoses in various complicated cases of these diseases.

The first prospective indepth immunological and immunopathological study of serum sickness in man has been completed. A previously unknown cutaneous sign in humans specific for serum sickness was described. The complement fragments C5a and C3a are thought to be important in the pathogenesis of the inflammatory response in cutaneous and systemic diseases. The C5a fragment has been purified and assessed for its in vivo and in vitro reactivity. The ability of C5a and C3a to modulate cell surface receptors for immunoglobulin and complement on the surface of leukocytes is being studied. Studies are in progress to evaluate the role of endothelial cells in immune complex mediated vasculitis.

A continuing project is the evaluation of the safety and effectiveness of new oral and topical agents, particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne. Oral etretinate has been shown to be effective and less toxic than isotretinoin in the treatment of the disorders of keratinization. In fact, isotretinoin has now been withdrawn from further clinical chemoprevention trials.

Cytogenetic studies are being conducted to determine if DNA-damaging agents induce abnormal numbers of chromosome aberrations in human cells which have a hypersensitivity to the lethal effects of such agents. A new cytogenetic test has been developed which enables the detection of hypersensitivity to DNA-damaging agents within a few days as an alternative to the currently used two to three week assay. Cells from patients with ataxia telangiectasia, tuberous sclerosis, Alzheimer's disease and other diseases are being studied. An abnormally high number of chromosomal aberrations were detected in ataxia telangiectasia heterozygote fibroblasts.

Ribonuclease H is an enzyme which hydrolyzes hybrid DNA (DNA:RNA). Hybrid DNA is apparently required for the initiation of DNA replication for semi-conservative DNA synthesis. Studies are in progress to determine if R-Nase-H is inducible by DNA-damaging agents, a positive finding of which would indicate a putative role of the enzyme in DNA repair. Capillary gas chromatography combined with mass spectrometry is being used in studies designed to detect and identify specific forms of damage to DNA.

One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. The keratin filaments are the most prominent intracellular component of all epidermal cells, and therefore, a study

of their chemistry, structure, and biosynthesis in both normal and abnormal epidermis will be of profound importance in studying tumors in this tissue. Two keratin genes: the mouse 59kD and human 67kD have been isolated and further characterized. Preliminary data suggest that these two genes and possibly those of other types have evolved from a common ancestor. Further detailed analyses of the genes and comparisons with other intermediate filament genes are in progress.

Metabolism Branch

The Metabolism Branch (Dr. Thomas Waldmann, Chief), conducts clinical and basic laboratory studies to define host factors that result in a high incidence of neoplasia, with a special emphasis on defining those factors involved in the control of the human immune response. A broad range of immunological investigations are carried out in patients with immunodeficiency diseases and a high incidence of malignancy, as well as in cancer patients, especially those with T- and B-cell leukemia. A second research goal is to determine the physiological and biochemical effects producing a tumor on the host metabolism. In this regard, the regulatory role of cell membranes, homeostatic mechanisms, and metabolic rearrangements of biochemical control mechanisms are being investigated.

Studies on the arrangement of immunoglobulin genes, and the rearrangements and deletions involved in the control of immunoglobulin synthesis have continued. The human immunoglobulin light and heavy chain genes, in their embryonic state, are organized in a discontinuous system of multiple germline regions. Human immunoglobulin (Ig) genes rearrange in an ordered fashion as pluripotent stem cells mature into immunoglobulin-synthesizing B cells and plasma cells.

During pre-B-cell development, heavy chain genes rearrange before light, and κ light chain genes generally precede λ. Recent work has shown that this ordered process includes an unanticipated deletion of the constant κ (C_κ) and κ enhancer gene sequences that precede λ gene arrangement. The recombinational element (Kde) responsible for this deletion has been cloned, and in subsequent studies it was found to recombine site specifically with a palindromic heptamer signal located in the J_κ -C_κ intron. All loses of C_κ genes in other pre-B cells were mediated by the Kde. In contrast, the Kde remained in its germline form on all successful κ-producing alleles. The Kde and its gene deletion are evolutionarily conserved as heteroduplex, and sequence comparisons reveal a high homology of this element between mouse and man. Thus, the κ -deleting element appears to insure isotypic and allelic exclusion of light chains and could mediate the ordered use of human light chain genes. This ordered sequence of immunoglobulin gene rearrangements has been exploited as tumor-specific clonal markers to follow the natural history of acute lymphoblastic leukemia (ALL). Moreover, the sensitivity of this approach has enabled the identification of clonal populations of cells in bone marrow during clinical remission in some patients with ALL several months prior to histopathologic evidence for relapse. Patients with B-cell follicular lymphomas frequently manifest t(14;18) (q32;q21) translocations. The chromosomal breakpoint was cloned from a cell line in which chromosome segment 18q21 was introduced flush with the J_{H6} joining region. This suggests that the same site-specific recombinatorial machinery which mediates DJ joining also mediates this interchromosomal translocation. The 18q21 element isolated mediates translocations in all four t(14;18)-bearing cell lines studied and in six of 11 unselected follicular lymphomas, but did

not normally rearrange in other B cells. Significantly, the location of breakpointe uniformly retained the Ig enhancer region in close proximity to the new transcriptional unit identified on chromosome segment 18q21. Regions of the genomic clone isolated from 18q21 recognize a 6.5- and 4.0-kb message within t(14;18)-bearing lymphoma cells. The cDNAs corresponding to these transcripts were cloned and it was shown that they have no close homology to known v-onc or c-onc genes. Thus, this element may represent a new transforming gene which, when introduced into the Ig heavy chain locus, may play a critical role in the altered growth or differentiation of the t(14;18) follicular lymphomas.

Previously, recombinant DNA technology was used to analyze Ig gene rearrangements to classify neoplasms previously of uncertain lineage, to aid in the diagnosis of neoplasms of the B-cell series and to define the state of differentiation of neoplastic B-cell precursors. Over the past year, a molecular genetic analysis of the genes encoding the T-cell antigen receptor has been undertaken to provide an analogous marker for T-cell clonality and lineage. These studies have culminated in the development of new approaches for the diagnosis and monitoring of the therapy of T-cell lymphoid neoplasia. All neoplastic expansions of T cells or their precursors, displayed an identifiable DNA arrangement. In virtually all cases the leukemic cells manifested multiple rearrangements identifiable as two new bands on Southern analysis of DNA from the malignant tissue not present in germline DNA. In contrast to the rearrangement in T cells, the majority of clonal B cells, as well as malignancies of nonlymphoid series, did not display rearrangement of the $T\beta$ gene, and in addition normal polyclonal T cells presented a pattern distinct from that of germline tissues or clonal leukemic T cells. Southern blot analysis of clonally rearranged $T\beta$ genes could detect even small minority populations of clonal cells within tissues admixed with normal cellular elements. An analysis of the $T\beta$ gene arrangement has been of value as adjunct in monitoring the therapy of patients with leukemias of the T-cell series. For example, in a patient with ATL expressing the Tac antigen, response to therapy was associated with a loss of the new band on Southern gel that is the hallmark of the clonal population. However, even before clinical relapse was observed, clonal cells with the rearranged band were again demonstrable with a $C\beta$ probe. During the final relapse, evidence of clonal progression was obtained since a new rearrangement of the $T\beta$ gene was demonstrated. This was paralleled by a failure of response to a previously successful anti-Tac immunotherapy. Thus, T-cell receptor rearrangements, in conjunction with studies of Ig gene rearrangements, aid in the definition of lineage (T-cells vs. B-cells) and the clonality of lymphoid populations of all series.

Another research goal has been to understand, and ultimately manipulate, the immune response at the antigen-specific level. The approach to this problem has been to study the immune response (Ir) genes, the structures of antigenic proteins required for activation of T lymphocytes, the interaction between monoclonal antibodies and defined sites on protein molecules, and the regulatory networks of antibodies recognizing specific combining site structures of these antibodies (idiotypes). Earlier work using sperm whale myoglobin as a model antigen demonstrated that there were two antigenic sites that were immunodominant for T-lymphocyte responses of a high responder strain of mice, and that each site was seen by T cells only in association with a particular major histocompatibility molecule ($I\text{-}A^d$ or $I\text{-}E^d$) on the surface of an antigen-presenting cell. Thus, in this system immunodominance depends strongly on the major histocompatibility molecule with which the antigen is presented.

Further studies demonstrated that both of the known immunodominant T-cell sites of myoglobin are amphipathic, suggesting that this must be a general property of sites antigenic for T_j cells. Additional evidence suggests that it may be possible to predict T-cell antigenic sites on the basis of sequence and secondary structure alone more easily than sites binding to antibodies. While the data have shown that T-cell antigenic sites are amphipathic structures, especially amphipathic alpha helices, the converse, that most amphipathic helices are good T-cell antigenic sites has not been conclusively demonstrated. This prospect, however, may prove to be a powerful predictive tool for locating T-cell antigenic sites which may be very useful for designing synthetic vaccines.

These studies have led to the discovery of two approaches to enhance or amplify weak antibody responses which may be useful for vaccines. Recently, it was found that incorporation of interleukin-2 (IL-2) in the adjuvant with the antigen at the time of immunization enhanced the antibody response of genetically low responder mice to the level of high responders. This mechanism may involve amplification of low levels of T-cell help and may be useful for immunization of immunodeficient patients. A second approach, utilizes the targeting of the antigen to the immune system by coupling the antigen to anti-Ig which can bind to B lymphocytes. This approach of enhancing immunogenicity may make possible successful immunization with very weak antigens or antigens available in only small quantities.

A major accomplishment of the Metabolism Branch has been the molecular cloning and characterization of T-cell growth factor receptor in normal and malignant T cells. Using hybridoma technology, a monoclonal antibody termed anti-Tac, was developed that identifies the T-cell growth factor receptor. Using this antibody, cDNAs encoding the human receptor for IL-2 were molecularly cloned, sequenced, and expressed in eukaryotic cells. The human genome contains a single receptor gene located on chromosome 10p band 14-15 and organized within eight separate exons. The mature IL-2 receptor protein is composed of 251 amino acids. This peptide is cotranslationally modified producing two precursor forms which are subsequently exported to the Golgi apparatus where O-linked sugar, sialic acid, and sulfate are added prior to display of the mature receptors on the cell surface. T-cell activation is characterized by an early rise and later fall in IL-2 receptor expression which is involved in the regulation of the T-cell immune response. The variable expression of IL-2 receptors is controlled, at least in part, at the level of receptor gene transcription. IL-2 receptors can be reinduced in "senescent" activated T cells, which have lost >90% of their receptors, by stimulation with antigen or mitogen, agents which activate protein kinase C and IL-2.

Utilizing anti-Tac, those reactions that require an interaction of IL-2 with its inducible receptor have been defined. Anti-Tac blocks T-cell proliferation induced by antigens, abrogates the generation of cytotoxic T lymphocytes but does not inhibit their action once generated, and inhibits the sequential development of late-appearing activation antigens on T cells. Recently, it was shown that anti-Tac inhibits concanavalin A, Epstein-Barr virus, and antigen-induced suppressor T-cell generation. Further, the specific antibody response to sheep red blood cells, rickettsia antigens, and haemophilus influenza antigens in vitro is inhibited by anti-Tac. Normal B cells could be activated to express IL-2Rs, to produce mRNA encoding the IL-2R, and to proliferate on addition of purified IL-2. Furthermore, B cells, T cells, and other lymphocyte populations manifest upregulation of IL-2Rs on addition of recombinant IL-2 to

cells expressing small numbers of such receptors. This upregulation of IL-2Rs required protein synthesis and RNA transcription but not cell division.

A monoclonal antibody, 7G7/B6, has been characterized which binds to the human IL-2R at a site distinct from its ligand, IL-2, and anti-Tac. Using 7G7/B6 and anti-Tac, an enzyme-linked immunosorbent assay (ELISA) was established to quantitatively measure soluble IL-2R. Using this ELISA, a released soluble form of the IL-2R was discovered in the culture supernatants of activated human cells in vitro. In addition, elevated levels of soluble IL-2R were found in the serum of certain patients with cancer and acquired immunodeficiency syndrome (AIDS). The elevated serum levels of IL-2R in cancer patients may inhibit the host's immune response to the tumor and thereby allow tumor growth. In addition, there were increases in the serum IL-2R following the administration of recombinant DNA-derived IL-2 to human cancer patients in vivo, which may limit the usefulness of this form of immunotherapy. Soluble IL-2Rs may play an immunoregulatory role, and the level of such receptors in the serum of certain patients may be indicative of an alteration in immune reactivity in vivo, and in addition, may provide a sensitive diagnostic test and a means of following therapy in patients with abnormalities of immune activation in vivo.

Human T-cell leukemia-lymphoma virus I (HTLV) infected adult T leukemia (ATL) cells uniformly express large numbers of IL-2Rs which occasionally may be aberrant in size. This amplified expression of receptors in ATL cells involves constitutive transcription of the IL-2 receptor gene and use of three (rather than two) promoters. The constant expression of large numbers of IL-2Rs which may be aberrant may play a role in the uncontrolled growth of ATL leukemic cells. A clinical trial to evaluate the efficacy of intravenously administered anti-Tac monoclonal antibody in the treatment of patients with ATL has been initiated.

The therapeutic studies have been extended in vitro by examining the efficacies of toxins and isotopes coupled to anti-Tac in selectively inhibiting protein synthesis and the viability of Tac-positive ATL leukemic cell lines. The most promising conjugate appears to be the pseudomonas exotoxin conjugates of anti-Tac. This conjugate will selectively inhibit the protein synthesis by an HTLV-I associated ATL Tac-positive cell line, and showed only minimum toxicity in cynomologus monkeys. Thus, the development of pseudomonas exotoxin conjugates of the monoclonal anti-Tac that are directed toward the IL-2R expressed on adult T-cell leukemic cells may permit the development of a rational approach for the treatment of this almost uniformly fatal form of leukemia.

Other investigators have had a long-term interest in the effects of the herpes viruses on the immune response in humans. Recent studies have focused on the nature of the immune response to cytomegalovirus (CMV). CMV presents a major problem in patients receiving organ or bone marrow transplantation and in patients with AIDS. Lymphocytes in culture respond to stimulation with CMV with impressive T-cell proliferation accompanied by B-cell activation and Ig synthesis. However, CMV induces Ig production only in cultures containing viable, unirradiated T cells, and only in lymphocyte donors who were CMV immune. Ultraviolet irradiated or heat-killed CMV was as effective as infectious virus in inducing these responses. These experiments indicate that CMV antigens are potent T-cell-dependent polyclonal B-cell activators and may help to explain some of the striking polyclonal B-cell activation and hypergammaglobulinemia commonly seen in patients with congenital or acquired CMV infections.

Another ongoing project has focused on the purification and characterization of immunoregulatory factors associated with human pregnancy. In recent studies a previously undescribed suppressor immunoregulatory glycoprotein, termed uromodulin, has been purified which appears to specifically block the in vitro activation of interleukin-1 (IL-1). Uromodulin is being characterized and may function not only in pregnancy but also as a regulator of normal immune responses. In related studies the in vitro immunoregulatory role of monosaccharides and complex oligosaccharides is being characterized. Two candidate oligosaccharides have been isolated that have marked in vitro immunosuppressive activity. These complex oligomers suggest a new class of immunosuppressive drugs.

Work has continued on the mechanism of action of insulin-like growth factors (IGFs), emphasizing an analysis of the receptors for these hormones. There are two cell surface receptors for the IGFs. The type I receptor binds IGF-I better than IGF-II, recognizes insulin weakly and is very similar to the insulin receptor structurally. The type II receptor prefers IGF-II over IGF-I, does not recognize insulin, and is structurally quite distinct from the type I receptor. Antibodies to the type II receptor are being developed which should be useful in sorting out the relative importance of the type I and type II IGF receptors in mediating particular biological responses associated with growth.

Like the insulin receptor, the type I receptor has been shown to have intrinsic tyrosine kinase activity directed toward the β subunit and extrinsic tyrosine-containing substrates, similar to the tyrosine kinase activity associated with the transforming proteins of retroviruses. No tyrosine kinase activity has been demonstrated in highly purified preparations of the type II receptor, either directed toward the receptor itself or against extrinsic tyrosine-containing substrate.

The synthesis of phosphoribosyl pyrophosphate (PRPP) and ribonucleotides is stimulated by pyrroline-5-carboxylate (P5C). This intermediate in the interconversions of proline, ornithine, and glutamate can activate the pentose phosphate pathway by a redox-dependent mechanism, thereby serving as a metabolic interlink between amino acids and ribonucleotides. Recent studies have focused on the physiological effects of this newly discovered regulatory mechanism. This effect of P5C has been shown to be synergistic to the effect of growth factors on ribonucleotide synthesis. Additional data suggest that P5C may mediate hormonal effects and, indeed, may act as a "primitive hormone." Such a role for P5C as an intercellular communicator has been supported by studies in normal human volunteers. P5C is found in human venous plasma, and, more importantly, the levels are responsive to nutritional perturbation increasing significantly following a protein meal. Thus, P5C may serve as a mechanism to link nutritional factors to growth factor action. Finally, P5C has been used as a probe to uncover redox abnormalities in tumor cells resistant to adriamycin. The transfer of oxidizing potential into the pentose phosphate pathway mediated by P5C is greater in adriamycin-resistant cells than in wild type cells.

Immunology Branch

The Immunology Branch (Dr. David Sachs, Chief), conducts studies of the regulation and control of immune responses, structure and function of cell surface molecules, immune effector cell mechanisms, transplantation biology, molecular biology, and tumor immunology.

A primary research focus has been the study of the regulation and control of immune responses. Previous studies have been extended to further analyze the mechanisms of regulatory T cells controlling B cell activation and antibody response. It was demonstrated that similar regulatory mechanisms control the activation of B cells by antigen-specific and major histocompatibility complex (MHC) restricted T helper cells or by autoreactive T helper cells. In addition the regulation of B cell idiotype expression was characterized in the T15-dominant response to phosphocholine employing cloned regulatory T cell populations.

The alloreactive T cell repertoire has been analyzed using large panels of cloned T cells. It was demonstrated that non-MHC as well as MHC encoded target determinants are recognized at high frequency by the population of autoreactive and antigen-specific T cells. There was cross-reactive recognition of Mls^a encoded determinants which was shown to be MHC restricted. This suggests that certain non-MHC determinants may play a critical role in the alloreactive T cell repertoire. Further investigations of the immune deficiency state which is induced by chronic graft versus host reactions (GVHR) have been carried out. The inability to generate cytotoxic T lymphocytes (CTL) is associated with loss of IL2 production, and loss of expression of IL2 receptors and helper T cell function. GVHR induced by combined class I and class II MHC allorecognition abrogated help for both self + X and allogeneic responses, whereas class II only allorecognition abrogated only responses to self + X, and GVHR induced by class I only MHC differences induced no immune suppression unless cytomegalovirus infection was concomitantly present. These studies are highly relevant to current clinical problems of GVHR induced in circumstances of bone marrow transplantation.

Earlier studies on immunosuppression have been extended to the investigation of humans at risk for acquired immunodeficiency syndrome (AIDS). Subjects at high risk for AIDS have decreased in vitro CTL responses to self + X antigens, but elevated T cell responses to HLA alloantigens. The data suggest that cofactors such as susceptibility to HLA alloantigenic stimulation may contribute to the development of AIDS.

Continuing studies have shown that two distinct classes of T helper cells participate in CTL responses against membrane bound antigens, and that only one of these T cell subsets functioned as helper cells in responses to minor histocompatibility antigens. T helper cells and T killer cells with identical Lyt phenotype and MHC restriction specificity differ significantly in their antigen response repertoires. Recent studies have shown that an $Lyt2^+$ inhibitory cell selectively blocks the generation of class II specific CTL, but not class I specific CTL, during in vitro sensitization culture.

A series of hybridoma antibodies has been produced which appear to recognize molecules selectively expressed on activated B lymphocytes. These molecules may be receptors for growth or differentiation factors. An analysis of the determinants on Ia molecules recognized by alloreactive, autoreactive, and antigen-specific T cells has been carried out. Cloned T cell populations responsive to given Ia molecules were differentially susceptible to inhibition by an extensive panel of monoclonal anti-Ia antibodies. These findings demonstrated the existence of multiple sites or conformations on an Ia molecule recognized by different T cells.

Studies of the functional role of T cell surface markers in human cytotoxic T cell recognition have continued. Effector T cells and target cells form non-specific as well as specific conjugates. The specificity of cytotoxicity appears to function at the level of T cell activation rather than binding alone. The T3 structure appears to be uniquely relevant to T cell triggering. Cytotoxic T cells have also been used to define phenotypic differences between lymphoblastoid cell lines which are not distinguishable serologically. This suggests the existence of previously unknown HLA encoded products or functions.

The human anti-mouse xenogeneic cytotoxic response has been used as a model system for the study of human alloantigen repertoire. These studies have provided information relevant to xenogeneic transplantation studies. It was demonstrated that human CTL exhibit a fine specificity capable of distinguishing alpha 1 and alpha 2 domain changes in the class I MHC molecules of murine target cells. A series of monoclonal antibodies specific for human T cell surface determinants has been generated. These monoclonals will be used to probe the biological functions of these antigens, and to eliminate T cells bearing these determinants from the marrow in an attempt to prevent GVHR in human allogenic bone marrow transplantation.

Studies of the molecular and cellular bases of the interactions of immunoglobulins with immune effector systems have continued. Both T cell and antibody dependent cell-mediated cytotoxic (ADCC) effector cells have been used to show that cross-linking of the receptors responsible for lysis to determinants on the target cell surface is sufficient to trigger lysis. This approach appears to be useful in evaluating the functional role of T cell receptor molecules in cytotoxic effector function. In addition, it represents a potentially useful means for targeting effector cells against tumor cells in models of immunotherapy.

In other studies, the biochemical characterization of the molecular components of cytoplasmic granules from large granular lymphocytes and cytotoxic T lymphocytes have been carried out to determine their role in the lytic function. The cytolyic capacity of these lymphocytes appears to be attributable to a single potent protein, cytolysin, which is capable of forming large membrane pores in the presence of calcium and lipid.

A major focus of research activity has been the analysis of structure and function of MHC products, and the manipulation of the immune response directed at these products. Studies of mixed allogeneic and xenogeneic chimeras, in which radiated animals are reconstituted with mixtures of T cell depleted donor and host-type marrow have been produced. It has been demonstrated that these animals are immunocompetent, and that tolerance is induced to donor-type MHC determinants in these animals. This represents an important potential model for the clinical application of allogeneic and xenogeneic organ and tissue grafts in the absence of graft rejection responses by the host.

Continued studies have been made to manipulate transplantation responses with anti-receptor antibodies in vivo and in vitro. This work has led to the production of antibodies directed at clonal receptor structures and other functionally related structures on T cells. Additional work has centered on the fine specificity of anti H-2 specific CTL. These studies have shown that cytotoxic T cells generated across limited H-2 differences detect determinants

distinct from those detected by monoclonal antibodies of similar fine specificity and can be used to define the structural determinants involved in MHC recognition by T cells.

Studies of transplantation biology using the inbred miniature swine model have continued. Two recombinants within the MHC have been detected, both of which involve separation of class I and class II encoding loci. Selected matching for class II antigens permitted long term kidney graft survival in 50% of animals tested, whereas skin grafts in the same combinations were rejected. Thus, class II tolerance may be particularly critical to allograft tolerance induction. A series of monoclonal antibodies has been prepared against swine T cell antigens, which are differentially expressed on porcine helper and cytotoxic T cells. These antibodies are being further applied to studies of T cell elimination in bone marrow grafting models in swine, where successful syngeneic reconstitutions of irradiated animals have been accomplished and where mixed allogeneic reconstitution experiments are in progress.

Additional studies have centered on examining the nature of the T cell subpopulations which function in in vivo graft rejection. It appears that the critical T cell subpopulation which initiates skin graft rejection is the IL2 producing T cell population, and that there are two subsets of such cells which can be distinguished by both their Lyt phenotype and their MHC specificity.

New procedures have been developed for T cell depletion from human bone marrow by treatment with monoclonal antibody plus complement. These depletion procedures produce minimal residual T cell contamination. Such T cell depleted marrows, characterized as to HLA type, extent of T cell depletion, viability, and stem cell progenator activity, are cryopreserved with the intent of application to clinical transplantation.

A porcine class I MHC gene has been isolated and introduced into the genome of the B10 mouse, where it is expressed on the surface of a variety of tissues. Skin grafts of such transgenic mice were rejected by normal B10 mice, suggesting that the foreign SLA antigen expressed on the mouse cell surface is recognized as a functional transplantation antigen. Further studies will be directed at T cell recognition of these xenogeneic gene products both as foreign MHC determinants and as "self" restricting elements.

The structure and genomic organization of class I MHC genes have been examined in the miniature swine. A novel class I MHC gene, which is only distantly related to the other members of the family, has been isolated and shown to be expressed in a variety of tissues. The regulation of expression of the class I gene families has been examined. A variety of regulatory regions were identified, and the positions of the transcriptional promotor and the interferon enhancer have been mapped. Sequence analysis of two class I MHC genes in the miniature swine has established that the structure of class I molecules in the pig resembles those of other species, and that the extensive polymorphism in this system is generated through variations confined primarily to the first and second protein domains of the molecule.

Recent laboratory investigations have been directed toward the isolation and characterization of genes which are transcriptionally regulated following mitogen and lymphokine activation of T-lymphocytes. It was hypothesized that genes induced within a few hours after antigen or mitogen activation of lymphocytes

will be fundamentally important for the initiation of proliferation and effector function expression in these cells. It was shown that the c-myc oncogene is transcriptionally induced as early as one hour after activation. Two additional proto-oncogenes, c-myb and c-fos, are similarly regulated by mitogen binding to the surface of lymphocytes. Initial results of studies using DNA mediated gene transfer techniques indicate that oncogene transfected cells generally show increased proliferative activity. Currently, the levels of mRNA resulting from the transcription of exogenously introduced DNA are being determined. In addition, subtractive cDNA libraries are being generated in order to isolate the set of genes activated by PHA stimulation of human peripheral blood T cells. These studies should provide a better understanding of the regulation and cell cycle associated role of oncogenes.

A long standing research study has been directed at understanding and manipulating the mechanism of cell-mediated immune cytotoxicity. Lymphokine induced cytotoxic cells (LICC) were demonstrated to function in vitro and in vivo in preventing growth of both lymphoid and solid tumors. Additional studies demonstrated that antitumor cytotoxic cell responses of murine cell stimulated in vitro by IL2 are modulated in a strain dependent fashion by particular macromolecular polyanions. Several of these substances occur naturally in man and may serve as regulators of IL2 activity. Human peripheral blood lymphocytes can be targeted to kill human tumor cells using anti T3 antibodies coupled with antibodies that detect specific determinants on tumor cell surface. Further studies will investigate the potency of these targeted effectors both in vitro and in vivo as models of tumor immunotherapy.

Laboratory of Immunobiology

The Laboratory of Immunobiology (Dr. Tibor Borsos, Chief), conducts studies on the interaction of antigens, antibodies and complement components, chemotaxis and inflammation, and on the immunological basis of tumor rejection in vivo.

A continuing focus of research is the binding, activation and action of antibodies and complement at cell surfaces. In the past year, the ability of mouse monoclonal antibodies to DNP to bind and activate complement has been studied. It was shown that the monoclonal antibodies to this hapten behaved essentially the same as the rabbit polyclonal Igs; indeed that monoclonal antibodies behave very much like polyclonal antibodies under conditions where the epitope distribution is optimal.

In studying the dissociability of anti-hapten antibodies as a function of hapten density and fluid phase hapten concentration, it was observed that removal of the antibody from the cell surface by fluid phase hapten was greatly reduced by an anti-antibody. To further study this phenomenon, a collection of anti-allotype antibodies and anti-antibodies was prepared against various domains in the anti-hapten antibodies. Results so far indicate that dissociability of b4b4 or b5b5 anti-hapten antibody decreased significantly when in combination with an anti-b4 antibody; however, the dissociability of the b4b5 anti-hapten antibody was barely affected by anti-b5 antibody. Since antibody-antibody complexes are highly C fixing and activating if such a complex is attached to a cell surface and is essentially non-dissociable it has a greatly increased chance to induce complement mediated damage than it could induce in more dissociable form.

The properties of complement sensitive and resistant tumor cells have been analyzed. Previous reports demonstrated that sensitivity of human, mouse, and guinea pig tumor cells to C mediated killing could be enhanced by pretreating the cells in vitro with selected metabolic inhibitors, and that in vitro grown human lymphoblastoid cells (Raji) and mouse mastocytoma cells (P815) vary in their sensitivity to C killing at various stages of asynchronous growth. Additional studies indicated that C sensitivity correlated with increased unsaturated fatty acids content, and, that the sensitivity of Raji and P815 cells could be manipulated by changing cell number or resuspending the cells in fresh culture medium. Recent studies have shown that C sensitive Raji cells contained a 130K dalton protein not contained in the resistant Raji cells, whereas C sensitive P815 lacked a 170K dalton and 16K dalton protein contained by the resistant P815 cells.

Recent studies suggest that, in addition to biochemical properties of the cell, binding, activation, and interaction of the early acting C components with C3 and/or with the late acting C components and the cell membrane may play an important role in determining C resistance.

A major research project is directed towards understanding the biochemical and genetic mechanisms by which immunogenic tumor escape from immune attack. Previous work suggested that within tumor cell populations variant cell populations develop that have lost cell surface transplantation antigens. Antigen loss appears to be an irreversible change in tumor cell phenotype and to be associated with tumor recurrence. Two models have been used to study this problem: retrovirus-infected fibrosarcoma cells, and melanoma cells transfected with a gene encoding a Class I antigen.

Previous studies showed that retrovirus infected fibrosarcoma cells grew, regressed, and then recurred in inbred guinea pigs. Studies showed that the tumor recurrences did not express retroviral cell surface antigens and were susceptible to superinfection with the homologous retrovirus. When genomic DNA was extracted from the cells that form recurrences it was demonstrated that these cells lack proviral sequences. Evidence suggests that the cells that form the recurrences have been selected from the infected cell populations.

Cells with partial, single or multiple copies of the provirus have been identified in the chronically-infected cell population. As might be expected, cells with a single copy of the provirus are at particularly high risk for genetic changes that would interfere with expression of retroviral cell surface antigens.

In another series of experiments, a plasmid containing a gene ($H\text{-}2D^d$) for a murine major histocompatibility complex antigen was transfected into the B16 melanoma ($H\text{-}2^b$) cell line. This plasmid was stable during passage of the transfected cell line in vitro. When B16 melanoma cells transfected with $H\text{-}2D^d$ were injected into syngeneic normal mice, the footpad tumors had the restriction pattern characteristic of the cell line; the pulmonary metastases lacked either the part or the entire plasmid. This represents another example where deletion of a gene encoding a cell surface antigen is associated with escape from immunological attack. Studies are underway to attempt to prevent tumor escape from immunological attack.

The analysis of human monocyte heterogeneity and chemoattractant binding by flow cytometry has continued. Studies confirmed that human monocytes and lymphocytes could be distinguished by different patterns of combined forward and 90 degree light scatter. However, it became apparent that these were two populations of monocytes, core monocytes, (within the monocyte window) and outlier monocytes (outside the window). In recent experiments, approximately 80% of core monocytes bound the chemoattractant fMet-Leu-Phe-Lys-FITC (FMLP), whereas about only 39% of outlier monocytes bound the ligand. Chemoattractant binding of the whole blood monocyte population was compared with migration to chemoattractant. Approximately 60% of all monocytes bound FMLP, whereas only 36% of monocytes responded to attractant by directed migration. Thus, lack of chemotactic migration among human blood monocytes can be accounted for by absence of receptors among one-third of total monocytes and absence of directed migration despite receptors in an additional one-third.

During the year, a study of chemotactic factors in Staphylococcus aureus culture fluids was initiated. Chemotactic activity comprised a series of peptides that ranged from 600 to 2000 daltons. It is significant that optimal concentrations of bacterial peptides attracted almost twice as many monocytes as the optimal concentration of FMLP. Studies are in progress to determine whether these differences are accounted for at the receptor level or subsequent to ligand-receptor interaction.

Studies of the mammalian serum protein (MSP) have continued. Previous work showed that MSP affected motility responses of mouse resident peritoneal macrophages, causing macrophages in culture dishes to put out long cellular processes, and to become responsive to chemoattractants. Further purification of this trace protein has been facilitated by the generation of a monoclonal antibody.

Laboratory of Cell Biology

A major focus of research activity in the Laboratory of Cell Biology (Dr. Lloyd Law, Chief), has been the isolation, purification, and characterization of tumor rejection antigens (TSTA). A new, mild ion-exchange chromatography step has been added to the purification process which enables the purified TSTAs to retain their in vivo tumor rejecting activity and specificity. Biochemical characterization of two purified TSTAs, p82 and p86 is underway. Efforts are currently underway to determine if they have different primary amino acid sequences and/or different post-translational modifications.

These sequence studies will be extended in an attempt to determine whether the individual TSTAs from a particular tumor have different primary sequences from that of their normal cellular counterparts or if the tumor rejecting activity is due to different subcellular distribution and/or other post-translational modification. With the information from the protein and DNA sequence analysis and tissue distribution of the TSTAs, it should be possible to elucidate the normal role of these proteins and the nature of their antigenic modification upon transformed state.

Past studies have shown that spontaneously induced murine melanomas, as well as those that are chemically or UV light induced produce TSTA which not only dis-

play autologous tumor rejection activity, but share determinants with the heterologous melanoma lines as well. Specifically studies show that this activity does not cross-react with any of the non-melanoma tumor lines, with the exception of neuroblastoma cells which have an identical embryologic derivation. Interestingly, a spontaneous pigmented melanoma, although highly immunogenic, does not appear to share cell surface TSTA with other melanoma lines examined. Purification of the TSTA associated with melanoma lines is now in progress.

Previous studies showed that tumor-specific proteins released from melanoma cells can be recovered from the serologic fluids of tumor bearing patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro. More recently it was shown that one of these shed proteins has structural homology to the albumins, and may represent the product of a normally occurring gene which is abnormally expressed in neoplastic tissues. Additional studies have shown that TSTA, isolated from sequential preparations of melanoma tumors growing in vivo, differed significantly in their amino terminal sequences. Thus, the variability in antigen expression may be responsible for the diversity of subpopulations of melanoma cells detected immunologically within a single tumor.

In previous work the T-cell immune response specifications of different natural and synthetic cytochrome c peptides of mice with different H-2 haplotypes were defined and T-cell clones specific for different cytochrome c peptides were established. These T-cell clones, along with antigen presenting cells, will be used in photochemical cross-linking experimemts designed to localize antigen during the different stages of T-cell antigen recognition.

The receptor for antigen expressed by mouse helper and cytotoxic T lymphocytes is composed of a disulfide-linked dimer. Genes encoding the α and β chains of this T cell receptor for antigen are rearranged and expressed in helper and cytotoxic T cell clones. In human T suppressor cell clones, the B chain genes are rearranged, whereas no somatic rearrangement of genes coding for the β chain has been observed in most mouse suppressor T cell hybridomas. Studies using a cloned T cell lymphoma line, obtained by radiation leukemia virus-induced transformation of hen egg-white lysozyme (HEL)-specific mouse suppressor T lympocytes have shown that at least some mouse suppressor T cells use the same set of genes as helper and cytotoxic T cells for their antigen-specific membrane receptor.

Laboratory of Tumor Immunology and Biology

The research activities of Tumor Immunology and Biology (Dr. Jeffrey Schlom, Chief), focus on the analysis of the biochemical mechanisms of oncogene expression and cell growth, the identification and characterization of cellular genes associated with the development of murine and human mammary neoplasia, and the development and utilization of monoclonal antibodies directed against tumor associated antigens.

One major research project investigates the biochemical mechanisms which may link oncogene expression to neoplastic cell transformation. It is thought that the activity of oncogene products will modify the synthesis, post-translational modification, subcellular location, or metabolic fate of one or more normal cellular proteins whose function is essential for normal growth control. One

approach has been to study the alterations in the synthesis of specific cellular proteins as a consequence of expression of retroviral oncogenes in murine (NIH/3T3) cells. Two proteins emerged from this analysis as the most promising common targets of retroviral oncogene action in the production of neoplastic transformation. These proteins were suppressed early during the appearance of the transformed phenotype, and were restored to normal levels of synthesis in both revertant lines studied. Further analysis determined that these proteins were components of the skeletal fraction and that they were tropomyosins. The synthesis and content of tropomyosins and other cytoskeletal proteins in various human cell lines and tissues is being investigated in order to determine whether synthesis of these proteins is also suppressed in human neoplasms. The following systems will be studied: lymphoid cells, myeloid cells, colon carcinoma, bladder carcinoma, and mammary carcinoma.

Studies of the mechanism of cell transformation and tumor formation by retroviral oncogenes in vivo and in vitro have continued. The current goal is to identify the cellular "target" molecules which are modified by the action of retroviral oncogenes, particulary ras, and thereby to begin to deduce pathways by which these oncogenes act.

The mechanism of transformation by retroviral oncogenes is also being studied through a biochemical analysis of transmembrane signal transmission systems which are known to be involved in cell growth regulation. It was previously shown that the activation of protein kinase C, a cellular receptor for phorbol ester tumor promoters, can be correlated with cell growth and transformation. Interleukin 2 causes a rapid, transient redistribution of protein kinase C from cytoplasm to nucleus in CT6 cells, and interleukin 3 has a similar effect on FDC-Pl cells. These results are evidence that the distribution and activity of protein kinase C can be modulated by growth factors.

Studies in genetics have focused on the characterization of the mouse mammary tumor virus (MMTV) genomes in inbred and feral strains of mice, and their potential role in tumorigenesis. Related projects have sought to identify cellular genes at risk in mammary tumors induced by infectious MMTV, and to characterize MMTV related sequences in human cellular DNA.

Studies of endogenous MMTV genomes in the BALB/c inbred mouse strain have demonstrated the expression of a novel of 1.6 kbp MMTV mRNA in normal lactating mammary gland and pristane induced plasma cell tumors. This species of MMTV mRNA is composed primarily of sequences from the long terminal repeat of the viral genome. The function of this protein in viral replication and the consequences of its expression in plasma cell tumors is being studied.

The activation of two cellular genetic loci (designated int-1 and int-2) by the insertion of an MMTV genome is associated with mammary tumor development in high incidence inbred strains of mice. One strain of feral mice which lacks endogenous MMTV genomes in its germline, is infected with an exogenous MMTV related virus. Examination of mammary tumors from these mice has led to the identification of a new common integration locus (int-3) for MMTV in tumor DNA, which is unrelated to the int-1 and int-2 loci as well as to other cellular oncogenes. It is located on mouse chromosome 17. The molecular and biological characterization of int-3 locus is in progress. In recent studies it has been shown that the MMTV gag-pol related sequences are located within a novel class of human endogenous retroviral genomes. Further studies are in progress in an

attempt to determine the contribution of this class of human retroviral genomes to the development of neoplasia.

Monoclonal antibodies have been developed to identify and characterize human carcinoma associated antigens and differentiation antigens of mammary and colonic epithelium. Of particular importance is the generation and characterization of an MAb (B72.3) that defines a novel tumor associated antigen (TAG-72). This MAb is selectively expressed in the majority of human colon, mammary, and ovarian carcinomas, and is currently being evaluated for use in the detection of occult tumor cells and the detection of carcinoma lesions in situ using radiolabeled immunoglobulin. Additional MAbs have been generated to a repertoire of epitopes on carcinoembryonic antigen (CEA) which are differentially expressed among carcinoma cell populations. In addition, MAbs to proteins associated with metastatic cell populations have been developed.

Analysis of ras p21 oncogene and proto-oncogene expression in human carcinomas is currently in progress. Monoclonal antibodies of predefined specificity have been generated by utilizing a synthetic peptide reflecting amino acid position 10-17 of the Hu-ras^{T24} gene product as an immunogen. The RAP (RA, ras; P, peptide) MAbs clearly define enhanced ras p21 expression in the majority of human colon and mammary carcinomas, while the majority of all abnormal ducts and lobules from fibroadenoma and fibrocystic disease patients were negative, as were the normal mammary and colonic epithelia examined. The RAP MAbs have been utilized to define ras p21 protein expression in a spectrum of colonic disease states. The data suggest that ras p21 expression is correlated with depth of carcinoma within the bowel wall, and is probably a relatively late event in colon carcinogenesis.

A variety of virally and chemically transformed cells and spontaneously arising tumor cells produce transforming growth factors (TGFs). TGFs confer upon normal cells several properties associated with the transformed phenotype and may be involved in the autocrine growth of these cells. Alpha-TGF, is structurally related to epidermal growth factor (EGF). Rat mammary tumor cells obtained from chemically induced rat mammary adenocarcinomas have a diminished response to EGF, which is probably due to the production of an alpha-TGF-like growth factor, termed mammary tumor factor (MTF). Chemically-induced rat mammary tumors have either an elevated expression or an alteration in structure of p21^{ras}. The potential role of TGFs in the transformation of NIH/3T3 cells has been examined. The studies to date suggest that the lesion(s) in flat revertants of ras transformed cells are distal to the elevated expression of p21^{ras} and production of TGFs and that the elevated production of TGFs is necessary, but may not be entirely sufficient for maintaining the transformed phenotype in ras transformed cells.

The presence and role of transforming growth factors (TGFs) in human mammary carcinoma cells is being defined. Relatively high levels of TGF activity were detected in crude, delipidated, decaseinated human milk. These levels of TGF varied among individual donors and were generally highest in colostrum. Three distinct TGF species can be detected in the human breast tumor samples with identical species found in human milk. One of the TGFs has been purified and partially characterized. This alpha-TGF species is virtually identical to the species found in the conditioned media of a human mammary carcinoma line, but is biologically and physiochemically distinct from the major species of human EGF in milk.

There is extensive heterogeneity in the expression of defined tumor-antigens both within human breast and colon carcinoma lesions as well as in established human tumor cell lines. This heterogeneity of tumor antigen expression can be found within defined areas of the tumor and within subcellular compartments of a single tumor cell. Factors such as cell cycle kinetics, long-term growth in vitro, clonal variability, and growth of tumor cells in three-dimensional structures appear to alter the antigenic phenotype of human tumor cells. The apparent ability of human tumor cells to intrinsically modulate their tumor antigen expression is an important factor contributing to this extensive antigenic heterogeneity. Recent studies have shown that recombinant human leukocyte interferon (IFNalpha A) can increase the binding of specific MAbs to the surface of human breast and colon carcinoma cells in a dose-dependent manner and thus mediate an increase in tumor antigen expression without any increase in tumor cell proliferation.

A monoclonal antibody (B72.3) has been generated using membrane enriched fractions of a metastatic human breast carcinoma as the immunogen. Previous studies demonstrated that the reactive antigen can be detected in tissue sections of human breast and colon carcinomas, and not in a variety of normal adult tissues. Using the avidin-biotin method of immunoperoxidase staining it demonstrated that MAb B72.3, selectively detects adenocarcinoma cells in effusion from patients with carcinoma of the breast, lung, and ovary. The clinical data suggests that MAb B72.3 may be applicable in the detection of occult tumor cells in needle aspirates and other body fluids.

Immunoglobulins from two MAb that bind to human breast and colon tumor associated antigens (B6.2 and B72.3) have been purified and F(ab')2 and Fab' fragments have been prepared from the B6.2 IgG. Recent studies have demonstrated that radiolabeled B6.2 IgG and fragments localize specifically in the breast tumor xenografts with the IgG giving maximal activity in the tumor. Model systems that resemble the metastatic nature of human colon carcinomas are being developed to better determine the efficacy of radiolabeled monoclonal antibodies as potential agents for radioimmunodetection and radioimmunotherapy.

Accurate detection and localization of both primary and metastatic lesions remains one of the major problems in the management of most human carcinomas. Clinical trials have been initiated using radiolabeled MAb B72.3 to detect and localize colorectal lesions. Various parameters are being investigated to assess the efficiency of MAb localization and the efficiency of gamma scanning of carcinoma lesions.

Another research project studies the mechanism underlying the control of growth of hormone-dependent mammary tumors. In previous work it was shown that cAMP antagonizes the estrogen action and produces growth arrest of hormone-dependent rat mammary carcinomas induced by DMBA. In the regressing tumors, the cAMP receptor level increased while estrogen receptor level decreased in a strictly inversed manner. These results were confirmed in a limited number of primary human breast carcinomas examined. This suggests that the determination of cAMP receptor levels may be a prognostic value for human breast cancer. Additional studies have sought to determine the effect of cAMP on gene expression in mammary tumors. In a series of experiments it was shown that in hormone-dependent rat mammary tumors, c-$\underline{ras}^{H}$ proto-oncogene is expressed in growing but not in regressing tumors. In early breast cancers in humans, it was shown that the elevation of p21 was strictly restricted in the estrogen receptor

positive tumors. The mechanism of hormone effect on p21 elevation in mammary tumors is currently being investigated.

Whether the growth regulatory effect of cAMP involves regulation of oncogene expression was investigated using a Ha-MuSV DNA transfectant of NIH-3T3 cells growing in a serum free defined medium. Treatment of cells with various cAMP analogs resulted in a marked decrease of p21 ras protein synthesis, which correlated with a change in the molecular species of cAMP receptor proteins, and with a change in morphology. Cells treated with cAMP analogs exhibited a morphology characteristic of untransformed fibroblasts, while the untreated cells retained a transformed phenotype. Thus, a role for cAMP and its receptor protein in the quantitative modulation of v-ras^H oncogene expression is demonstrated. The mechanism of this cAMP action at genomic level is currently being investigated.

Previous laboratory studies suggested that production of basement membrane proteins is important for the growth of normal mammary epithelium and the adenocarcinomas arising from it. A growth factor (MDGF-1) that stimulates proliferation of normal and neoplastic mammary cells has been purified from human breast tumors and from human milk. This factor interacts with high affinity membrane receptors and stimulates the cellular production of basement membrane collagen and laminin. A series of proline analogs have been analyzed for their effects on collagen synthesis inhibition in cultures of primary DMBA-induced rat mammary tumors and for their effects on mammary tumor growth in tumor-bearing animals. Three compounds were found that inhibit collagen synthesis and can cause tumor regression or growth arrest in vivo. A positive correlation appears to exist between the ability of a tumor to synthesize basement membrane and its proline analog concentrations which affect tumor growth. Blocking basement membrane depositions, thus favoring tumor cell contact with stroma, may promote proline analog uptake and tumor cell kill. In contrast to primary tumors, metastatic rat mammary tumor growth was not affected by proline analogs. Using cDNA clones against type IV collagen, it was demonstrated that metastatic tumors lack collagen IV mRNA sequences, suggesting that metastatic cells may fail to produce a basement membrane because they have lost the ability to produce mRNAs for laminin proteins. Thus, the lack of mRNA species may prove to be a useful marker for metastatic potential.

Studies have been designed to understand the role of hormones, vitamins, and growth factors in normal mammary gland development and differentiation. The purpose of these studies is to define the conditions involved in normal and hyperplastic development of the gland and production of mammary achieved growth factors. Additional experiments have been undertaken to evaluate the nature of the interaction of lactogenic hormones with their receptors. The stability of the hormone-receptor complex, and the nature of the cryptic sites is being investigated, as well as the effects of alterations in membrane lipids and membrane aggregation. Purification of the lactogenic hormone receptors is underway in order to prepare antibodies for studies on regulation of synthesis of the molecule.

Galactosyltransferases play an important role in the synthesis of cell surface determinants in normal and neoplastic cells. Recombinant DNA technology is being used to isolate and characterize a galactosyltransferase cDNA clone which will provide a useful tool for studies in the regulation of expression of this

gene in normal and neoplastic cells. Galactosyltransferase activity is modulated by α-lactalbumin (α-LA)-like molecules. Studies are in progress to determine the structure and sequence of this gene. This information should provide a better understanding of this modifier activity of this protein towards galactosyltransferase and also hormonal regulation of the expression of this gene in normal and neoplastic transformation of the mammary cells.

SUMMARY REPORT

LABORATORY OF GENETICS, DCBD, NCI

October 1, 1984 through September 30, 1985

The laboratory has continued to operate as a cooperative group of independent scientists whose common theme is the study of how specific genes determine and regulate a variety of biological functions relevant to the problems of neoplastic development. The laboratory is supported by a contract facility maintained at Litton Bionetics, NO1-CB-25584, which raises and supplies most of the mice and tumors used in the lab. The laboratory meets weekly from September to May to discuss work in progress and on a separate day with Dr. John Minna's group to conduct a seminar on oncogenes. The laboratory sponsored one workshop on the "Novel Immunological Responses of the BALB/c Mouse," held on March 11-12, 1985. The meeting was a stimulating conference that brought into focus a number of intriguing new insights in mouse genetics. The location of our laboratory in Building 37 has facilitated a number of collaborative projects.

The induction of plasmacytomas in BALB/c mice by the intraperitoneal injection of pristane or mineral oils provides a model system for studying the pathogenesis of a neoplasm. This year we have found that abnormal proliferations of plasma cells in the pristane-induced oil granuloma can appear as soon as 25 days after the introduction of pristane. Many are present by day 50, i.e., months before fully malignant transplantable tumors develop. These lesions provide cells in intermediary stages of neoplastic development and will allow us to study the role of susceptibility-resistance genes, growth factors, mutagens, chromosomal translocations and other biologically active mediators on this phase of plasma cell tumor progression. We have already obtained evidence that indomethacin can inhibit these proliferations at day 60.

Dr. Richard Nordan has been isolating a growth factor, PCT-GF that is required by many plasmacytomas for growth in vitro. In collaboration with Dr. Stuart Rudikoff he has begun purification of this factor. Dr. Nordan has found plasmacytoma cell lines that absolutely require this factor for survival.

The genetic basis of susceptibility to plasmacytoma development in BALB/c mice has not been determined but sustained efforts to find responsible genes are continuing. These depend upon the development of susceptible-resistant congenic pairs of mice. The BALB/cAn (susceptible) BALB/cJ (resistance) system offers special advantages because this may be an example of a near coisogenic susceptible resistant pair.

While numerous phenotypic differences have been described between these two BALB/c sublines, no relevant genotypic differences have been found. Dr. Konrad Huppi has isolated through gene cloning, DNA probes that distinguish BALB/cAn from BALB/cJ. He is developing a variety of methods based on DNA subtraction-hybridization for this purpose.

Dr. J.F. Mushinski has been studying the structure and expression of two protooncogenes, myc and myb, which are involved in plasmacytomas and ABPL

lymphomas. These oncogenes, expressed in normal cells and tumors and are overexpressed in certain inherited autoimmune diseases. A major effort is to study the regulation of myc and myb expression. Somatic cell hybrid studies in collaboration with C. Croce et al., have established for the first time the orientation of the 3 mouse c-myc exons and the c-sis gene with respect to the centromere of chromosome 15 and corrected the published Ig gene orientation of V_H, C_H and c-fos on chromosome 12. The new concept that an important myc control sequence resides as close as 350-500 bp 5' of c-myc has evolved from studies of 6 plasmacytomas in collaboration with K. Marcu et al. In this work it was found that translocation interrupts the c-myc gene just 5' of myc exon I. Further studies on myc expression are focusing on TEPC1165 and TEPC2027, which are superproducers of c-myc RNA probably owing to both a uniquely marked stimulation of rate of c-myc transcription and a diminished rate of myc mRNA breakdown. Similar studies of c-myb transcription are in their infancy compared to c-myc but we know that in ABPL tumors transcription is up-regulated by insertion of Mo-MuLV near the 5' end of mouse c-myb. Contrariwise, in certain mouse myeloid tumors (induced by Dr. Herbert C. Morse) helper virus also integrates into the myb locus and causes premature termination of transcription and abnormally small myb mRNA. Efforts will be intensified by DNA sequence analysis to localize the site of rearrangements for both these oncogenes and to understand their effects on gene transcription. Two new approaches are about to be utilized toward these ends: expression and antiexpression vectors (in the form of retroviruses) will be constructed to determine directly the biological effects of over- or under-production of c-myc or c-myb products. In addition, in situ hybridization of cytoplasmic c-myc and c-myb mRNAs will be utilized in collaboration with Dr. Mary Harper, to identify when and where rcpt(12;15) occurs and which cells exhibit dysregulation of expression of these oncogenes.

Stuart Rudikoff's laboratory has been studying gene evolution in natural populations with an intent to define mutational mechanisms that operate on a variety of immunologically important genes. Recombinant DNA libraries have been contructed from six wild mouse species and have been screened with probes corresponding to the κ chain constant region, a κ chain V region family and the β chain of the T cell receptor. Homologous genes have been isolated from all species, and those from M. pahari have been sequenced. A comparison of these sequences from those of inbred mice reveal specific patterns of evolution associated with each gene. The two constant region genes of the T cell β chain appear to have been "corrected" by processes such as gene conversion resulting in coding regions which are much more homologous than would be expected by classic evolution. This process of correction appears to have occurred independently in both inbred mice and M. pahari. In contrast, the coding region of the Cκ gene reflects the accumulation of point mutations in a manner expected of single copy structural genes. However, the 5' flanking region, as well as the enhancer region, are as conserved as the coding region. The pronounced conservation in the 5' flanking region is unexpected and may indicate additional function associated with this portion of DNA sequence. The Vκ gene family exhibited a complex pattern of evolution suggesting possible interchange of gene segments among family members and indicating that these genes are clearly subject to different mutational processes than pauci-gene families even in the relatively short evolutionary period of the species Mus. In summary, each of the genes Dr. Rudikoff's group has examined

appears to be evolving in an independent manner and subject to different mutational processes. This finding is important to our understanding of the mechanisms involved in the introduction of polymorphisms in natural populations which subsequently effect corresponding phenotypes. Furthermore, the data point out the difficulty in attempting to make generalizations about evolution of genes, i.e. biological clocks, in that marked variation is found among gene segments which belong to the same "superfamilies" and perform putatively similar functions. Dr. Rudikoff is now developing a series of projects to isolate and characterize genes controlling growth factors, and growth factor receptors.

Dr. Michael Rogers' laboratory is studying the antigenic structure of tumors and the origin of cell surface tumor-associated antigenicity. His work focuses on a novel antigen found on most lymphomas induced by the Friend, Moloney and Rauscher retroviruses (FMR antigen). In collaboration with Dr. Louis Matis, Dr. Rogers has made considerable progress in the characterization of the gp175 FMR antigen isolated from the Rauscher virus induced T-cell lymphoma RBL-5, by isolating specific T-cell clones. The sensitivity and specificity of this assay will now make it possible to develop DNA probes to identify the gene(s) that control gp175 and determine the genetic basis for the origin of the antigenicity. Dr. Rogers' group is continuing to study unusual antigens derived from MHC genes, Q10 and Qa2.

Dr. Sandra Smith-Gill has determined the primary structure of the monoclonal antibody, HyHEL-10 to hen egg white lysozyme (HEL) and constructed a hypothetical model of this protein. Using a scaled model to HEL, a complementary structure to the putative HEL-epitope has been found on HyHEL-10. The HyHEL-10 epitope is now being more precisely mapped by data obtained from heavy and light chain recombination studies done by Dr. Smith-Gill in collaboration with Dr. Keith Dorrington. Sequences of closely related V_L and V_H regions have been employed in some of these recombinations. These data coupled with the comparative binding activities of the recombinants for different avian lysozymes are fine-tuning this system. Work is proceeding in collaboration with Dr. David Davies to determine the three dimensional structure of a crystal of HyHEL-10 Fab combined with HEL. Work is proceeding on generating via site specific mutagenesis a system for producing and expressing further variants. Hopefully this work should provide a basis for designing antibodies with predetermined specificity.

Drs. Sandra Ruscetti and Linda Wolff are continuing their investigations in the mechanism by which murine leukemia viruses induced erythroid transformation. Comparisons of the molecular structure of two variants, $SFFV_P$ and $SFFV_A$, and formation of a recombinant virus have allowed localization of a region within the envelope gene that determines the ability of infected cells to differentiate in response to erythropoietin. Other experiments have shown that the long terminal repeat (LTR) region of SFFV, unlike Friend murine leukemia virus, does not carry sequences required for tissue-specific induction of leukemia, since a number of different LTRs can be substituted without affecting the disease latency or phenotype. And finally, the generation of helper-free SFFVs using a packaging cell line has provided proof that helper virus is not required for <u>in vitro</u> transformation of erythroid cells, first stage acute erythroleukemia in mice or develoment <u>in vivo</u> of second stage tumorigenic cells.

Studies on the genetics of susceptibility to early erythroleukemia induced by Friend murine leukemia virus have identified a gene on chromosome 5, at or near the Rmcf locus, that plays a major role in resistance of mice to this disease by preventing the replication of mink cell focus-inducing (MCF) viruses, believed to be the proximal cause of the disease. This gene is believed to be either a structural gene or a regulatory gene for an MCF virus-related envelope glycoprotein that appears to block the cell surface receptor for MCF viruses. Additional genes, acting through unknown mechanisms, may also be involved in resistance.

Dr. Hayden Coon has continued his studies of cultured cloned normal thryoid cells, and in collaborative experiments with Eugene Bell at MIT has been studying the capability of these cells to reconstitute thyroid function in thyroidectomized rats. Dr. Coon plans to continue studying problems related to reconstruction of tissues from cultured cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 CB 05596-16 LGN

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pathogenesis of plasma cell neoplasia: characterization of antigen-binding proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.:	M. Potter	Chief, Laboratory of Genetics	LGN,NCI
	E. B. Mushinski	Bio. Lab. Tech.	LGN,NCI
	L. D'Hoostelaere	Biologist	LGN,NCI
	E. Shacter	Staff Fellow	LGN,NCI
	K. Huppi	Staff Fellow	LGN,NCI
	K. Sanford	Chief, In Vitro Carcinogenesis Sect.	LCMB,NCI
	S. Brust	Biologist	LGN,NCI

COOPERATING UNITS *(if any)*

Dr. H. C. Morse, III, NIAID; Dr. Francis Wiener, Karolinska Institutet, Stockholm, Sweden; Dr. A. O. Anderson, Mucosal Immunity Lab., Aerobiology Division, USAMRIID; Dr. Jo Hilgers, Netherlands Cancer Institute, Amsterdam; Dr. Ram Parshad, Howard U.

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	4	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major project in the laboratory is to determine the pathogenetic mechanisms involved in the development of plasmacytomas in BALB/c mice. Plasmacytomas are induced by the intraperitoneal injection of pristane; their formation depends upon the genotype of the host (BALB/c and NZB inbred mice are susceptible); the chronic inflammatory process initiated by the phagocytosis and containment of pristane and by the generation of internal mutagenic substances emanating from the chronic inflammation (oxygen and lipid radicals). The work involves: 1) the identification of genes that determine susceptibility and resistance to plasmacytoma development through the construction of congenic pairs of mice; 2) a study of the inflammatory process to identify biologically active factors in plasmacytomagenesis (growth factors, mutagens), and 3) a detailed study of the histopathogenesis of plasmacytomas to determine the time and site of origin of the chromosome 15 translocations and the progressive changes in plasma cells leading to the autonomous state (proliferative focus development).

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

I. Identification of genes that determine susceptibility of resistance to plasmacytomagenesis in BALB/c mice

Professional Personnel:

The project is supported by NCI Contract NO1-CB-25584 which maintains and operates a closed, conventional colony of inbred and wild mice protected by a quarantine. The mice are bred in the same conventional facility where the induction study is carried out. This is an essential requirement to prevent the development of intercurrent infections and to obtain reliable data for the 300 to 360 days of the induction study. Judith S. Wax is a co-principal investigator on this contract. In house, Dr. Konrad Huppi, NCI, collaborates on this project by identifying genetic differences using DNA subtraction hybridization.

Objectives:

To identify, enumerate, locate and characterize genes that determine resistance (R-genes) or susceptibility (S-genes) to plasmacytomagenesis in BALB/c mice.

Methods Employed:

BALB/c is highly susceptible to the induction of plasmacytomas (PCT) by the intraperitoneal (i.p.) injection of pristane or mineral oils (i.e., 60% develop PCTs in one year), while most conventional strains are resistant. In addition, within the BALB/c family of sublines we have found that the BALB/cJ mouse is resistant to plasmacytoma induction. Specifically, we are trying to identify the R-genes from strain DBA/2, BALB/cJ and STS.

The first steps in identifying R genes is to locate them on a chromosome and to determine the phenotypes (functions) they determine or regulate. Since the genomes of inbred strains differ at many loci, one of the approaches is to find congenic pairs that differ by only PCT-S and PCT-R genes.

1. DBA/2

A first approach in identifying DBA/2 R genes was to determine if PCT-R genes are linked to known markers, or relevant phenotypic characteristics that distinguish DBA/2 from BALB/c. A series of BALB/c.D2 semicongenic stocks were constructed by backcrossing D2 markers or phenotypes onto BALB/c for 6 backcross generations. Over 10 such stocks have proved not to contain a linked PCT-R gene. Several of these stocks, i.e., those carrying $Fv\text{-}1^n$ (chr 4) and Qa2 (chr 17) have shown a weak partial resistance. One candidate for a strong R gene effect is with a stock bred for the gene Tol-1 phenotype. Unfortunately the assay for this phenotype is erratic and unreliable; however, fortuitously N8 progeny have been obtained and these have shown evidence of strong resistance. We are attempting to find a genotypic correlate by screening these mice against a wide variety of discriminating probes and also to develop

a probe by subtraction hybridization.

The major experimental problems in accomplishing this goal is identifying resistant mice by an induction assay. This process requires 250 days. However, we have a new potential method for identifying R mice by using preplasmacytoma focus assay. In the last year we have found a possible method for identifying susceptibility by examining a large sample of peritoneal oil granulomatous tissue during the latent period of plasmacytoma formation. Quite surprising has been the finding that proliferative foci containing atypical plasma cells can be identified as early as 25 days after the injection of pristane. Experiments are in progress to quantitate the number of foci/mouse as a function of time in order to find a minimal time for discriminating between S and R phenotypes. Although this work is in progress, preliminary studies have shown its feasibility at day 200. Using this method we have identified "low foci" mice at day 200 and have advanced our BALB/c.D2 PCT-R congenic to N3.

2. BALB/cJ

The resistance of BALB/cJ has been confirmed by the focus assay method where it has been found that BALB/cJ develop far fewer persisting foci by day 200. Detailed studies of the chronic inflammatory tissues in BALB/cJ in collaboration with Dr. A.O. Anderson have revealed several other interesting morphological differences, a decreased angiogenic response in BALB/cJ, a decrease in the intensity of the inflammatory process, and an increase in the lymphocytic infiltration of the oil granuloma. BALB/cJ is known to have most of the same allelomorphic genes as in other BALB/c lines including BALB/cAnPt; there are, however, a number of phenotypic differences, none as yet have relevance to tumor formation. One of the phenotypes is the Raf-1^b allele (a recessive gene) that determines a high serum level of alphafetoprotein in adult life. We are constructing a BALB/cAnPt.BALB/cJ Raf-$1^{b/b}$ congenic to determine if Raf-1^b is linked to resistance. BALB/cJ has at least 5 other phenotypic differences from other BALB/c sublines, and it has been suggested by Leslie Kozak of the Jackson Lab that BALB/cJ may have a major regulatory gene mutation that has multiple pleiotropic effects. We obtained preliminary evidence that Raf-1^b is linked to a second phenotype the 3rd component of Mup-1 (Mup.1^{+3^-}), a trait discovered by T.H. Roderick of the Jackson Lab. BALB/cAnPt is Mup1+3+. This should permit us to accelerate the development of BALB/cAnPt.BALB/cJ congenic. Studies are in progress to determine if these mice are susceptible to plasmacytoma development. Preliminary studies suggest that (AnPt x J)F_3 Raf-1^{bb} are resistant.

3. Defect in DNA repair (collaboration with K. Sanford, R. Parshad)

We are pursuing the BALB/cJ-AnPt difference by looking for other relevant phenotypes relating to DNA repair. Specifically in collaboration with Drs. Katherine Sanford and Ram Parshad we are attempting to identify G2 DNA repair difference. BALB/cAnPt has been shown to have a delayed G2 DNA repair in a study that has been in progress for over a year, while DBA/2 has an effective

G2 repair mechanism. Specifically, this will be adapted to study the differences between LPS-blasts (B-lymphocytes) in BALB/cJ and BALB/cAnPt.

4. Subtraction hybridization to find genomic differences in congenic mice (collaboration with K. Huppi)

In collaboration with Dr. Konrad Huppi, DNA subtraction hybridizations have produced DNA probes that distinguish genomic DNA from BALB/cAnPt and BALB/cJ. The first of these, the 19 probe detects multiple restriction fragment bands. This new approach should provide a number of new markers.

5. CXS STS mice (collaboration with J. Hilgers)

We are determining the susceptibility of STS, and 14 strain CXS recombinant inbreds developed by Jo Hilgers. Dr. Hilgers has been able to map a large part of the mouse genome based on differences between these two strains. We have bred 7 of the strains on the contract facility and have begun the testing. This long experiment promises to be a very rewarding effort, but can be potentially accelerated by the focus assay.

Significance to Biomedical Research and the Program of the Institute:

Many forms of neoplastic development, including viral, chemical, physical, and unexplained models have a genetic influence that determines susceptibility or resistance. Very few of these systems are condusive to genetic analysis, but the induction of plasmacytomas in BALB/c mice offers a useful experimental system. First, the inducing agents in question are mineral oil, pristane or plastics that are metabolically inert. Second, the process of a sporadic, somatic cell retroviral infection with the usual generation of recombinant forms as in AKR leukemogenesis appears not to be a contributing factor. These facts suggest the BALB/c plasmacytomagenesis model lacks the stigma of retroviral infection, and is not dependent on a known exogenous mutagenic agent.

The identification of specific genes, and their path of action in an experimental model of tumor development should elucidate critical events in neoplastic development but also identify genotypes that are at higher risk for tumor development. This may provide critical information that could be extrapolated to certian forms of human tumor development.

The availability of congenic pairs of mice that differ by susceptibility resistance genes will provide a practical system at the organismal level, and in addition, the relevant cells from PCT-S and PCT-R mice which can be used to study the action of these genes.

II. Development and Progression of Plasma Cell Tumors in BALB/c Mice

Professional Personnel:

This project is supported in part by the NCI contract NO1-CB-25584. A collaborative study with Ms. Susan Brust (NCI) and Dr. Francis Wiener (Karolinska) on cytogenetics of foci is also a part of this work.

A. Objectives:

To determine the specific steps in plasmacytoma development.

B. Project Description

In the last year we reported that the non-steroidal antiinflammatory drug indomethacin drastically inhibits pristane-induced plasmacytomagenesis. In searching for possible explanations I examined the oil granulomas during the latent period of plasmacytoma development in pristane, and pristane-indomethacin treated mice and found that indomethacin treatment inhibited the development of proliferative plasma cell foci in the oil granuloma. These observations were made at days 116 to 180 post pristane.

A method was developed to obtain the entire mesentery en bloc and to sample these tissues throughout the latent period of plasmacytoma development. A surprising finding was the fact that focal proliferations of plasma cells in the oil granuloma containing atypical cells were found very early. A substantial number of mice 25 to 50 days after the injection of pristane, i.e., often over 60 days before the minimal latent period of 120 days for clinical tumors, appearance and much longer than the mean latent period of 200-220 days. The following experiments are designed to study these preplasmacytoma lesions.

Methods:

Mice are injected with 0.5 ml pristane i.p. on days 0, 60 and 120. Peritoneal tissues removed en bloc after removal of the intestine are fixed, then fragmented in small pieces and examined by standard H and E slides and methyl green pyronin stained sections. Slides from different groups are randomized and then each slide is scanned and examined for foci of proliferating plasma cells. The number of foci are determined. Immunohistochemical techniques will be used to determine if the foci are monoclonal, and to determine the relationship of foci to each other in cases where there are multiple foci. Elizabeth Mushinski has developed these assays in our laboratory.

Indomethacin (20 µgm/ml) is administered in the drinking water to certain experimental groups.

1. Kinetics. The histogenesis of plasmacytic foci P.F. in BALB/c mice at days 25,50,75,100,150 and 200 is in progress to determine the number of mice with foci, per time period, the number of foci per mouse, and to correlate the morphology of these foci with plasmacytoma development. In addition, mesenteric node Peyer's patches and spleen are also examined. Since 60% of BALB/c plasmacytomas express IgA we will be very interested in looking for precursor populations.

2. Strain differences. Development of P.F. in C57BL/6, DBA/2, CDF_1, BALB/cJax, STS and congenic mice should allow us to determine if BALB/c plasma cells differ from those in other strains.

3. Effect of indomethacin. The effect of indomethacin on the quantitative kinetics of (P.F.) development is in progress. Indomethacin inhibits P.F. between 116-180 days. It will be important to determine if these effects can be detected earlier.

4. Separation of lymphoid and plasma cells from D.F. We are attempting to isolate proliferating plasma cells from oil granulomas and with Dr. Francis Wiener of the Karolinska Institutet, will try to obtain karyotypes on these cells. The major question here is, can we detect chromosome 15 translocations in day 50 samples? If so, this would suggest that myc gene deregulation is an early event in mouse plasmacytomagenesis.

Ms. Susan Brust is developing isopyknic cell separations in Percoll gradients to extract dividing plasma cells from the oil granuloma. She will use this method to make chromosome spreads to determine when the translocations appear.

5. Effect of transforming viruses on P.F. development. In collaboration with Dr. Herbert C. Morse, NIAID, we are testing the effects of transforming viruses on P.F. development. Studies are in progress on viruses that contain myc-raf oncogenes.

6. Transfer of P.F. cells. A major problem in studying the preplasmacytoma cells in P.F. is the inability to culture these cells, and to determine their neoplastic potential. Primary plasmacytoma cells depend on the pristane-induced oil granuloma microenvironment for growth. We have transferred peritoneal exudate cells to pristane conditioned mice without success. If however, the cells lie within the fixed tissue (oil granuloma) successful transfer may be accomplished. Accordingly oil granulomas from 50 day BALB/c mice are being transferred to pristane conditioned BALB/c x DBA/$2F_1$. While CDF_1 mice are resistant to developing plasmacytomas they rigorously support the growth of primary BALB/c (growth dependent) plasmacytoma cells.

Major Findings:

1. We have found that foci can appear as soon as 25 days after the injection of pristane and that a substantial number of BALB/c mice have foci by day 50.

2. Indomethacin inhibits focus development by day 60.

Significance:

Understanding the process of neoplastic development in an experimental system may lead to an understanding of correlative mechanisms in human carcinogenesis. A particularly interesting facet of this model system is the fact that in over 95% of karyotyped plasmacytomas in BALB/c a chromosome break involving the c-myc oncogene locus has been found.

An increasing list of human tumors have now been found to be associated with non-random translocations, e.g., CML, Burkitt's Lymphoma, other B-cell lymphomas and others. The plasmacytoma model systems offer a system for dissecting out relevant factors in neoplastic development.

A major problem in many forms of tumor development is understanding the process of tumor progression. The plasmacytoma model offers an experimental approach to this problem. The availability of the plasmacytic foci should allow us to determine if susceptibility to abnormal proliferation is genetically determined at the target cell level. In addition the oil granuloma system is itself a potential source of mutagenic substances, e.g., the toxic oxygen molecules liberated by macrophages and neutrophils. These exogenous radicals can potentially attack B-lymphocyte plasma membranes and cause the formation of lipid peroxide radicals which are released in the target cell. Several other highly relevant models of tumorigenesis or mutagenesis are 2-cell systems that have an oxygen-radical generating cell and a separate target for neoplastic transformation of mutagenesis. Chronic inflammation has been postulated to be a factor in certain forms of human neoplasia.

III. Genetics of immunoglobulins in mice (L. D'Hoostelaere, LGN, NCI)

The Ig kappa light chain locus has been studied in this laboratory for many years. Mr. D'Hoostelaere is attempting to order the VK genes in relation to the centromere.

Objectives:

The murine Ig-K locus is on chromosome 6 approximately 32 centimorgans from the centromere. The complex locus consists of approximately 90 to 320 variable kappa (VK) region genes spaced on the average 10 kilobase pair (kbp) apart, five joining (J) region genes, and a single constant (C) region gene. Although extensive studies have been made on this complex locus, little is known about the internal organization of the VK genes, and the orientation of the locus with respect to other genes on the chromosome has not been established. I have chosen to determine the order of selected VK genes on chromosome six using a combination of nucleic acid techniques and classical genetics. The genetic crosses will be made in a fashion which will allow an ordering of these VK genes, and their relative position with respect to CK and other selected genes on chromosome six.

Major Findings:

Production of the CK^a-Hd recombinant mouse resulted in a gene order centromere-CT β -3.4 Cm-Hd-4.5 CM-CK. Analysis of the Igk recombinant mouse "NAK" indicates Lyt 2.3; Ef-1; VK4; VK8, VK10; VK19 and VK21 are on one side of the recombination event and Ef-2; VK11 and VK24 are on the opposite side of the recombination event. Examination of inbred strains suggest a gene order of VK21-VK11-Ef-2-VK24.

Significance to Biomedical Research and the Program of the Institute:

There are separate coding regions for the two classes of light chains, kappa and lambda. Since over 97% of the Ig molecules formed in inbred strains of mice have kappa light chains, the number of variable kappa (VK) coding genes could be considered an important component of the Ig diversity in the mouse. These studies will provide additional information concerning the organization of

this multigene family and could be used as a model for comparison with other multigene families. The organization of this complex locus could lead to speculations about the methods of gene duplication.

Proposed Course of Research:

The use of the morphological markers wa-1 and Hd on chromosome 6 which flank the Igk complex are detectable at 2-3 days of age and can be used to reduce the number of progeny to be tested by 90%. These markers have been mapped distal and proximal to the Ig-k locus respectively. Preliminary examination of the inbred strains which express the wa-1 and Hd markers does not show adequate REF differences using the CK and VK probes. In order to make use of these morphological markers it will be necessary to produce populations which carry VK REF pattern differences with wa-1 and another having the unique CK REF with Hd. The Hd marker will be used in conjunction with the unique CK REF of SJL. The wa-1 marker will be used in conjunction with the VK REF differences of AKR mice.

The ABP.AK-VK^a/VK^a-wa-1/wa-1 (1st recombinant) have been mated to the S.C.B6.C3-Hd/+ mice. The f_1 progeny which expresses the Hd phenotype will be backcrossed to the 1st recombinant parental population. The progeny which express both or lack both outside phenotype markers will be recombinant mice, and DNA from these mice will be tested for recombination within the Ig-k locus using the CK and VK region probes. Since all of the REF of the AKR mouse will be present in at least one copy in all of the N_1 progeny, only the presence or absence of unique REF can be detected. This will allow a mapping of the majority of the unique inbred REF.

An additional cross involving ABP/Le-wa-1/wa-1 and C_{58}-Hd will allow a mapping of the other allelic forms of VK genes. Seventeen genomic clones have been isolated for 4 VK gene groups. Additional clones will be isolated from other VK groups and these clones will be used in an attempt to find flanking region polymorphisms which can be used in the mapping studies.

A cDNA library is being screened to obtain clones for VK gene groups which are not available at present. To date 40 clones have been isolated and analysis is beginning.

IV. Mediators of inflammation and their potential role in murine plasmacytomagenesis (Emily Shacter)

Objectives:

Plasmacytomas (PCTs) are induced in genetically susceptible strains of mice by agents that provoke a chronic inflammatory response. Substantial evidence indicates that development of PCTs is dependent upon factors in the microenvironment created by the peritoneal chronic inflammatory tissue. The studies described herein address two key questions presented by this system.

First, how do factors produced in the inflammatory tissue [e.g., polypeptide growth factors (GFs), prostaglandins, oxygen radicals] promote abnormal B-cell/plasma cell proliferation? To examine this problem, PCT cell lines that are dependent upon or independent of macrophage-derived GFs are being employed to study the biochemical mechanisms through which one such factor, PCT-GF, stimulates cell proliferation. Numerous metabolites (e.g., cyclic nucleotides, phorbol ester) are being tested for their capacity either to replace PCT-GF activity or to inhibit cell proliferation in the presence of PCT-GF. In addition, direct biochemical analysis of the immediate effects of PCT-GF on intrcellular parameters (e.g., protein phosphorylation, cyclic nucleotide levels) are being carried out. We will also adapt PCT-GF-dependent and -independent cell lines to growth in serum-free medium to facilitate accurate analysis of the possible interaction between PCT-GF and other factors (e.g., transferrin, inhibitors) present in serum.

The second question being addressed is how B-cells from genetically susceptible and resistant strains of mice respond differently to the damage to DNA induced by high concentrations of oxygen radicals present in the inflammatory tissue. Recent evidence suggests that strains of mice that are resistant to pristane-induced plasmacytomagenesis (e.g., BALB/cJax, DBA/2) may have more efficient mechanisms of DNA repair than mice that develop PCTs (e.g., BALB/cAn), and poly-ADP-ribosylation of nuclear proteins has been implicated in this process. We will test the hypothesis that the biochemical basis for genetic susceptibility to plasmacytomagenesis lies in the differential capacities of B-lymphocytes from susceptible and resistant strains to carry out ADP-ribosylation reactions that are critical for DNA repair. To this end, sensitive methods will be developed to study specific poly-ADP-ribosylation and any associated reactions in proliferating B-lymphocytes from BALB/cJax and BALB/cAn mice. Then, cells will be subjected to mutagenizing levels of oxygen radicals and known mutagenic agents to correlate mutagenesis, levels of ADP-ribosylation, and DNA repair.

Methods Employed:

Culture two plasmacytoma cell lines, TEPC 1165 and MOPC 460D, which are, respectively, dependent upon and independent of a polypeptide growth factor, PCT-GF, isolated by Richard Nordan of this laboratory. Treat the cells with various bioactive compounds in the presence and absence of PCT-GF and measure proliferation by ^{3}H-thymidine incorporation. Adapt the cells to culture in defined serum-free medium by testing different media and supplements. Study intracellular

levels of protein phosphorylation in response to PCT-GF by labelling cells with ^{32}Pi and identifying target phosphoproteins and phosphoamino acids by standard methodologies. Prepare B-lymphocyte LPS-blasts from splenic lymphocytes and label endogenous poly-ADP-ribosylated proteins with ^{3}H-adenosine. Develop enzymatic and cellular oxygen radical generating systems.

Major Findings:

1. Preliminary biochemical results indicate that PCT-GF probably does not act directly via activation of protein kinase-C or stimulating Ca^{2+}-influx, nor can it be replaced by cAMP or cGMP. Instead, the data suggest that cAMP produced endogenously and dibutyryl cAMP added exogenously are anti-proliferation factors for these cells, and that one initial response of dependent cells to PCT-GF may be the diminution of intracellular cAMP.

2. MOPC 460D and TEPC 1165 cell lines were adapted to growth in chemically-defined serum-free medium. The only proteins added to the cultures are BSA, transferrin, and insulin. As TEPC 1165 cells growing serum-free are still dependent upon PCT-GF, it can be concluded that PCT-GF stimulates growth by acting directly on the cells and not via interaction with unknown serum factors.

Significance to Biomedical Research and the Program of the Institute:

Artificial induction of inflammation and plasmacytomagenesis in the peritoneal cavities of genetically susceptible strains of mice provides an excellent model system for studying the biochemical basis for susceptibility to disease and the roles of inflammatory agents in promoting B-cell neoplasia. Moreover, employment of cultured plasmacytoma cell lines and primary B-lymphocyte cultures provides a means for elucidating these complex biological problems under relatively controlled experimental conditions.

Proposed Course of Research:

a) Identify, isolate, and characterize the biological activity of the putative membranal receptor for PCT-GF. b) Identify specific poly-ADP-ribosylation reactions and develop methods to clarify their involvement in preventing tumorigenesis.

DNA Hybridization Subtraction: Differences Detected Between Mouse Congenic or Inbred Strains at the DNA Level (Konrad Huppi)

Objectives:

Following the development of various inbred strains of mice, the use of congenic lines has become a valuable method for selection and enrichment of inherited phenotypic or genotypic traits. The region of DNA from potentially any phenotypic or genotypic marker can be placed on a different genetic background by the construction of a mouse congenic line. Since the context of this region of DNA in the congenic strain of mouse differs in some respect from the same region in the parental strain, subtraction methods can be applied to isolate and clone this DNA. We have adopted this approach to isolate and identify by recombinant DNA methods various traits which have been established in mouse congenic lines in our laboratory over the past several years.

Our laboratory has been primarily involved in investigating the onset of plasmacytomagenesis following treatment of mice with plastic materials, mineral oils or pristane. One mouse inbred strain, BALB/cAnPt, appears to be more susceptible to plasmacytoma induction than other closely related sublines such as BALB/cJax. One genotypic difference has been determined between BALB/cAnPt and BALB/cJax sublines, the expression of the major histocompatibility complex associated locus Qa-2,3. A few other phenotypic differences between BALB/cAnPt and BALB/c Jax include levels of alfa-fetoprotein expression, levels of enzymes involved in the catecholamine biosynthesis pathway and gdc-1, a marker associated with brown fat accumulation around the neck. Thus, it appears that BALB/cAnPt and BALB/cJax sublines are very similar to congenic lines of mice in that they differ by only a few traits among a vast background of identical allelic markers.

By the application of DNA hybridization subtraction, we have begun a systematic search for probes which recognize DNA differences between BALB/cAnPt and BALB/c Jax. In addition to the known allelic differences between these strains, we intend to characterize and map, chromosomally, new differences found at the DNA level. The association of particularly unique DNA hybridization patterns to "plasmacytoma susceptible" congenic strains of mice is of particular interest in this project. For example, a congenic line of mice, BALB/cJax. BALB/c AnPt has been established and selected for the locus raf-1 associated with alfafetoprotein levels. This congenic is also useful in plasmacytoma induction studies and will be tested for correlation with DNA hybridization patterns. Furthermore, a series of recombinant inbred lines (Bailey RI) have been established using C57BL/6J and BALB/cByJ mice and tested for susceptibility to plasmacytomagenesis. DNAs have been made from these lines and are available for studies with DNA probes.

Major Findings:

Utilizing the ability of homologous DNA to reassociate following denaturation, we have succeeded in cloning probes which recognize differences between subtracted DNA samples. One category of DNA probes which we have obtained map to mouse chromosome Y as a result of BALB/c male by female hybridization subtraction. These probes (Jax43, Jax118 and tol-20) represent useful markers

for mouse chromosome Y which, heretofore has been somewhat devoid of markers. In genetic analysis using these Y chromosome associated probes, an occasional phenotypic female mouse will contain hybridizable fragments. Previous literature indicates that genetic material from mouse chromosome Y is capable of crossing over to chromosome X. The results from our DNA probes substantiate that a female breeder mouse contains genetic material originally derived from the Y chromosome. Genomic cloning and sequence analysis of this DNA indicates this region of DNA is highly conserved among mouse species as a multicopy sequence. Further studies intend to examine transcription of this region with respect to tissue specificity and developmental status.

A second category of DNA probes which we have obtained recognizes restriction fragment length polymorphisms (RFLP) between BALB/cAnPt and BALB/cJax for autosomal loci. One particular probe π19 appears to recognize regions of DNA which are found in BALB/cAnPt and not in BALB/cJax. The probe π19 hybridizes to two DNA fragments of about 22 kb in size upon Bam HI digestion of BALB/cAnPt DNA. In contrast, BALB/cJax displays only one Bam HI band of 22 kb in size when hybridized to π19. Since π19 also recognizes differences between the mouse inbred strains STS/J and BALB/cAnPt, we have begun to chromosomally map this probe through a series of recombinant inbred (RI) lines established by Dr. J. Hilgers of Amsterdam. By Southern analysis of Bailey RI lines, raf-1 congenics and various other BALB/c sublines, we know π 19 shows no correlation with plasmacytoma susceptibility, raf-1 expression or Qa-2,3. We intend to compare π 19 to other phenotypic differences and to examine possible expression of this DNA.

A third category of DNA probes also recognize RFLPs but hybridize only to genomic DNA from which the probe was isolated. One example, π 71, hybridizes to an EcoRI fragment of about 10 kb in size in DNA from BALB/cAnPt but no detectable hybridization is evident in BALB/cJax. We have just begun to analyze this probe and future comparisons will help to characterize this region of DNA unique to BALB/cAnPt. Among the 200 DNA probes tested to date, about 10-20% of the probes recognize RFLPs between BALB/cAnPt and BALB/cJax.

Significance to Biomedical Research and Program of the Institute

We have developed a method of subtraction hybridization of genomic DNA which is capable of cloning virtually any region of DNA which differs among closely related individuals. This technique is especially applicable to cloning regions of DNA associated with phenotypic or genotypic markers from congenic strains of mice. Similarly, closely related BALB/c sublines which differ by only a few traits are particularly interesting in that they differ by their apparent susceptibility or resistance to plasmacytomagenesis. By assuming this difference between BALB/cAnPt and BALB/cJax sublines is found at the genomic DNA level, the method of DNA hybridization subtraction may ultimately lead to DNA probes involved in the resistance or susceptibility to plasmacytomagenesis. As a by-product of this technique, we are also accumulating low copy number DNA probes which map to different chromosomes of the mouse.

Proposed Course of Research

We are continuing to characterize DNA probes which recognize differences between BALB/cAnPt and BALB/cJax at the genomic DNA level. The strategy of approach includes the following: 1) Determine RFLP between BALB/cAnPt and BALB/cJax. 2) Compare RFLP among "susceptible" and "resistant" strains of mice with respect to plasmacytomagenesis. 3) Compare RFLP among congenic lines established for other markers such as raf-1, gdc-1, Qa-2,3. 4) Map the DNA probe to a chromosomal position and determine presence or absence of RNA transcript. 5) Finally, if the DNA probe shows correlation with a particular phenotypic trait, constructs can begin to be made to integrate this DNA into strains of mice lacking this trait.

We are also collaborating in similar DNA hybridization subtraction experiments to isolate DNA probes associated with various markers from other mouse congenic lines. Some of the traits which we are investigating are ity^R; a marker associated with resistance to parasite infection such as Salmonella typhimurium (in collaboration with D. Weinstein and A. O'Brien); gld-1, a chromosome 1 trait associated with autoimmunity (in collaboration with J. Mountz); SCiD-1, a severe combined immunodeficiency trait which affects C.B-17 mice (in collaboration with W. Schuller and M. Bosma) and tol-1, an immunological variation in tolerance to BCG associated with BALB/c mice with respect to DBA/2 mice (in collaboration with P. Schricker and C. Cowing).

Publications:

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Mainhart, C.R., Potter, M., and Feldman, R.J.: A refined model for the variable domains (Fv) of the J539 β -(1,6)-D-galactain-binding immunoglobulin. Mol. Immunol. 21: 469-478, 1984.

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Shen-Ong, G.L.C., Potter, M., Mushinski, J.F., Lavu, S., and Reddy, E.P.: Activation of the c-myb locus by viral insertional mutagenesis in plasmacytoid lymphosarcomas. Science 226: 1077-1080, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08727-08 LGN

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Organization and control of genetic material in plasmacytomas

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, leboretory, and institute effiliation)*

P.I.:	J. F. Mushinski	Medical Director	LGN,NCI
	G. L. C. Shen-Ong	Visiting Fellow	LGN,NCI
	K. Huppi	Staff Fellow	LGN,NCI
	S. R. Bauer	Biologist	LGN,NCI

COOPERATING UNITS *(if any)*

Kenneth Marcu, Dept. of Biochemistry, State Univ. of NY, Stony Brook, NY
Carlo Croce, Wistar Institute, Philadelphia, PA
J.D. Mountz, A&R, NIADDKD; H. C. Morse, LVD,NIAID; D. M. Klinman, A&R, NIADDKD

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	2.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It is the long range purpose of this project to study the control mechanisms important in regulating cell growth, neoplastic transformation, and protein synthesis in normal and malignant cells of the immune system. To this end we are studying the structure of the proto-oncogenes in normal and tumor tissues from mouse and man and the expression of these oncogenes as mRNAs. In particular we are focusing on mouse plasmacytomas, myelogenous tumors and lymphosarcomas, and we are investigating what role Abelson and Moloney leukemia viruses play in the induction of such tumors and the alteration of cellular ongogenes. We have discovered that these viruses induce a morphologically distinct subset of tumors (ABPLs) which have altered myb mRNAs owing to the insertion of a deleted form of Moloney leukemia virus in the myb gene. This represents a mammalian example of oncogene activation by promoter/enhancer insertion of virus. The ABPL tumors now appear to be of myelogenous origin, so we are studying the myb genes in other mouse myelogenous tumors. The expression of c-myc is increased in plasmacytomas, and in certain plasmacytomas with chromosomal translocations just 5' of the myc exon I, RNA transcription utilizes the two myc promoters in a different ratio than in normal cells. Two of these plasmacytomas are super producers of myc RNA and are being studied for clues to the mechanism of this enhanced RNA synthesis. We are also studying the expression of oncogenes in mouse and human autoimmune diseases, as well as their expression in normal lymphocytes activated by mitogens. Certain classes of autoimmune diseases are characterized by high levels of myc and raf RNA, while others have elevated levels of myb RNA.

Project Description

Objectives:

The major objectives of our studies are to understand the organization and regulation of genes important in neoplasia and in normal and abnormal immune processes. More specifically, we are concerned with elucidating the extent of involvement of oncogenes in neoplasia, autoimmunity and normal cellular growth and development.

Methods Employed:

Our experimental model systems include mouse plasma cell tumors, lymphosarcomas, myeloid tumors and autoimmune tissues. DNA and mRNA are extracted from tumors that are maintained in inbred mice or in tissue culture. Human tissues are primarily tissue cultured lines or peripheral blood cells. The DNA is studied by Southern blotting, genomic cloning, heteroduplexing and DNA sequencing. The mRNAs are studied by electrophoresis, blotting, S1 nuclease mapping, nuclear run on studies, R looping, cDNA cloning and DNA sequencing. Both plasmids and bacteriophage vectors are used for cloning and propagation of DNA clones in bacteria and for production of hybridization probes.

Major Findings:

A. Our studies of the DNA structure and RNA expression of proto-oncogenes such as c-*myc*, c-*myb* and c-*abl*, in tumors such as mouse plasmacytomas has continued. We have extended our original observation of rearranged *myc* gene DNA in pristane-induced plasmacytomas with 12;15 chromosome translocations to show that exons II and III of the *myc* gene are translocated from chromosome 15 to chromosome 12 ($12q^+$) in plasmacytoma J558 and not vice versa. This was demonstrated using somatic cell hybrids between Chinese hamster fibroblasts and J558 cells. One subclone contained only the $12q^+$ mouse chromosome. This subclone contained the structural exons of c-*fos* and c-*sis*, the rearranged *myc* gene and the structural exon for the "aberrantly" rearranged IgA α heavy chain. This clone did not contain Ig heavy chain variable region genes nor *myc* exon I and its 5' flanking region; these are presumed to be located on the reciprocal counterpart of this translocation, i.e., the $15q^-$ chromosome. This establishes for the first time that the mouse rcpT(12;15) occurs in the same manner as it occurs in human Burkitt's lymphoma. This is not what would have been predicted by the published mapping of mouse chromosome 12. This experiment disproves the published gene order and instead establishes the order as follows: centromere - c-*fos* - C_H - V_H for chromosome 12 and centromere - *myc* exons I-II-III - c-*sis*.

We have concentrated our studies of the expression of the c-*myc* gene on the effects of rcpT(12;15) which break the *myc* close to the 5' boundary of that gene but not within the gene itself. We are studying six examples of such tumors and find that all six have several structural and functional characteristics in common. There is a surprisingly restricted area between 350 and 500 base pairs 5' to *myc* exon I, where the breakpoints in all these tumors occur. In the two tumors in which the breakpoint has been pinpointed by DNA sequencing,

the myc gene is broken at precisely the same place, 361 bp 5' of exon I. Different tumors have different regions from chromosome 12 5' to this point of breakage and recombination with the myc gene of chromosome 15, but all of them are Ig heavy chain switch regions ($S\gamma_{2a}$, $S\gamma_{2b}$, two with Sμ and two with Sα). Some of these recombination events seem to be more complicated than merely a simple rcpT(12;15) but include an additional event, such as an inversion or an insertion of an isolated piece of another region of chromosome 12, often the enhancer-containing Sμ region. The functional consequence of all these 5' recombinations is the same, regardless of the nature of the switch region involved or the presence of the heavy chain enhancer region. S1 nuclease protection studies indicate that the myc mRNA from all these tumors has been transcribed starting at the two normally used promoters (P1 and P2). In these tissues P1 is used more often than P2, reversing the usage ratio (P2 > P1) found in normal organs and tumors lacking the interruption of myc 5' to P1.

Two of this group of plasmacytomas (TEPC 1165 and TEPC 2027) are especially remarkable in that they are super producers of myc mRNA, containing approximately 20-30 times the amount of myc RNA found in normal spleen. In addition, they produce myc RNA of an unusually large size. S1 nuclease studies and genomic cloning of the TEPC 1165 myc gene indicates a deletion of ca. 200 bp at the 3' end of myc exon I. Northern blot analysis indicates that the large TEPC 1165 myc RNA contains sequences from the first intron, undoubtedly due to the mutation which deleted the donor splice site at the 3' end of exon I. It is likely that some similar mechanism is operating in TEPC 2027, because its large myc RNA also contains intron sequences. Cloning of this myc fragment is being attempted to resolve this point.

Other studies of TEPC 1165 are underway in order to understand what mechanisms are responsible for its unusually large amount of myc RNA. Initial studies of myc mRNA stability indicate that TEPC 1165 has a half life in excess of 8 hours, a remarkable increase compared to the published myc RNA half life of 10-15 minutes in normal cells. Since the work of our collaborator, Ken Marcu, suggests that myc mRNAs truncated by chromosomal recombinations which interrupt myc exon I or intron I also have prolonged half lives, it appears that the presence of intronic sequences in the mature myc mRNA significantly stabilizes the message. Early experiments measuring the rate of myc RNA transcription by nuclear run-on experiments show that TEPC 1165 may be more actively transcribed than any other cells studied in a large series investigated by Marcu and his colleague, Marc Piechaczyk. Thus, TEPC 1165 offers many clues to the normal and abnormal regulation of myc RNA levels. TEPC 2027 with similarly high myc RNA levels but some structural differences should help us identify the stability enhancing and transcription stimulating alterations in TEPC 1165 by comparison with those found in TEPC 2027.

B. We have continued to study several different aspects of the ABPL tumors which we have earlier described as having altered myb DNA restriction fragments and unusually large and abundant myb RNAs. Based on FACS studies of surface antigens and biochemical analyses of cloned cells, these tumors appear to be in the myelo-monocytic lineage. This fits with literature reports that myb RNA

changes are more frequently seen in myeloid cells, including myeloblasts from which the v-myb containing virus, avian myeloblastosis virus (AMV), are isolated. Although we still have not identified all the 5' exons of mouse or human c-myb, we feel that all ABPL tumors have a deleted helper virus inserted just 5' to the c-myb exon homologous to the 5' end of v-myb.

We observed earlier that a Cas-Br-M virus-induced myelogenous leukemia from the laboratory of Dr. Herbert Morse produced an unusually small myb mRNA. Southern blotting now indicates that the c-myb gene in this tumor is rearranged at an exon close to the 3' end of v-myb. The rearranged fragment has been cloned and shown to contain the Cas-Br-M virus. This suggests that the regions immediately 5' and immediately 3' of the v-myb-like c-myb exons are especially vulnerable to viral integration, much like the way AMV is thought to have arisen from recombination of helper virus and certain central portions of the avian c-myb gene. In the mouse tumors, viral integration in the 5' area of c-myb leads to abnormally large and abnormally abundant myb RNA transcripts. In those with virus integration the 3' area of c-myb, the amount of myb RNA is not excessive, but the size is unusually small, probably owing to premature termination of RNA transcription in the interrupting viral LTR.

C. Studies on the expression of oncogenes in normal, resting, mitogen-stimulated, or autoimmune lymphoid cells and B lymphocytic tumors have continued. We have now accumulated Northern blots which describe the patterns of expression of many of the oncogenes in both mouse and human tissues of these types. It is not yet possible to summarize these findings into broad generalized interpretations. It is possible to make certain statements which have important implications.

1. Various strains of mice can be made to develop autoimmune disease by introducing the lpr/lpr (lymphoproliferation) gene in a homozygous form. The cells in the lymphadenopathic organs characteristic of this disease are filled with abnormal T cells characterized by abnormally large amounts of myb RNA. The thymus of these diseased mice contain abnormally low amounts of myb RNA. Both these abnormalities are reversed and the autoimmune disease is ameliorated by treatment with cyclophosphamide. We think this indicates that lpr/lpr disease is mediated by a defect preventing immature T lymphocytes (high in myb RNA) from stopping in the thymus for maturation and deletion of antiself reactive cells. Instead, they pass through the thymus incompletely processed and make their own way to the periphery where they participate in autoantibody production.

2. Normal human peripheral blood leukocytes (PBL) can be stimulated to divide by T- and B-lymphocyte mitogens. After such stimulation myc, myb and abl RNAs rise in B cells and T cells, but myb RNA does not rise in B cells. Curiously, fos RNA is high in PBLs before and after separation into B- and T-cells, but after mitogen stimulation, the fos RNA levels rapidly fall. This suggests that circulating PBLs are not in Go state when circulating, and the high fos RNA levels suggest that they are competent to be stimulated to divide.

3. B cell lymphomas of different degrees of maturity have different amounts of oncogene RNA. In general, it seems that the oncogenes that are found intranuclearly, myc, myb, fos and p53, have higher levels of RNA in immature (pre-B-cell) tumors, and less in mature B cell malignancies. This may mean that end stage B-cells (i.e., normal plasma cells) have very little RNA for these

oncogenes, but plasmacytomas with substantial amounts of myc RNA may have been rendered immortal or transformed by the presence of an atypically large amount of myc mRNA.

Significance to Biomedical Research and the Program of the Institute:

The goals of this laboratory and our parts of the Program of the National Cancer Institute are directed toward the understanding of the genetic and molecular events that result in cancer. The two types of oncogenes have been unambiguously implicated in oncogenesis in cancers caused by oncogenic retroviruses (v-onc) and in chicken bursal lymphomas caused by insertion of a non-oncogenic (helper) retrovirus near the 5' end of the c-myc oncogene. Normal c-onc genes are known to be important growth regulating factors in normal cells as well, so increased knowledge of the mechanisms controlling their expression would be valuable in our quest to understand these normal processes and how the abnormal processes in cancer cells lead to malignancy. This report details how chromosome translocations that disrupt the myc gene 5' to exon I lead to dysregulation of c-myc expression in plasma cell tumors. In addition, it seems that increased stability of myc mRNA also plays an important role in increasing the c-myc RNA levels in tumors of this type.

The hamster-mouse hybrid data presented here demonstrate for the first time in the mouse the gene order for Ig heavy chain genes and the 3 c-myc exons on their respective chromosomes.

The myeloid tumors that have helper virus integrated have distinct evidence of promoter/enhancer insertion in the 5' area of the c-myb oncogene leading to abundant transcription of an abnormally large mRNA. At the 3' end of the gene viral integration seems to lead to premature termination. So here are two instances of how non-oncogenic viruses can interact with and alter the expression of important, cancer-related oncogenes. The evidence is not yet conclusive connecting increased expression of oncogenes such as myc and myb with carcinogenesis. Nonetheless, it may be fair to extrapolate our oncogene expression data to conclude that terminal stages of differentiation are generally characterized by low levels of RNA from the genes of myc, myb, fos and p53. Thus, the plasmacytomas and myeloid tumors may have developed in part as a result of the abnormal levels of myc and myb RNA, respectively.

Proposed Course of Research:

We are intensifying our studies into the detailed structural alterations in (1) plasmacytomas with both chromosomal breaks 5' to myc exon I and defects in splicing of myc intron I, and (2) myeloid tumors induced by the same regimen but with helper virus insertions in the myb locus. The structural alterations will be correlated with expression abnormalities by careful studies of myc and myb RNA production using S1 nuclear mapping of promoter sites, nuclear run on studies of RNA polymerase transcription activities at these genes and RNA stability studies. Further studies on the normal structure of the very large and complex c-myb gene will be required, so efforts to isolate cDNAs that include 5' myb sequences are continuing.

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Another approach to correlate oncogene expression and induction of tumors or autoimmunity will be the construction (currently underway) of expression vectors that can be inserted into animal cells to increase the levels of myc, myb or other oncogenes and follow the biological consequences. Expression vectors that synthesize mRNA homologous to the noncoding strand of structural DNA will also be constructed with the important potential of decreasing the synthesis of myc and myb protein in normal, tumor and autoimmune cells. Another critical reagent, lacking so far, is active antibodies to mouse myc and myb protein products. These are neded to assess whether the tumors that have superabundant RNAs encoding these products also produce abundant protein product. Expression vectors will be used in an attempt to produce enough polypeptide to immunize rabbits, rats and mice to these oncogene proteins

Another new approach of the highest priority will be initiated by in situ hybridization of cytoplasmic RNA with oncogene probes. If this technology can be worked out, we hope to be able to determine when and in what cells high levels of oncogene RNAs are synthesized: (1) in incipient plasmacytomas induced by pristane; (2) in Abelson virus treated animals that will develop different types of hematopoietic tumors; (3) in autoimmune lymphatic organs before and after treatment; (4) in cells transfected with expression vectors, etc. We have arranged a collaboration with Dr. Mary Harper, NCI, who is an expert in this technique. We have prepared the appropriate "riboprobes" that she recommends for in situ hybridization, so we hope to begin this approach very soon.

The search for a second oncogene mutation in plasmacytomas will concentrate on the ras family of genes. Alterations in the DNA of these genes and their expression in plasmacytomas and related tumors will be studied. Abnormalities in src or other protein kinase genes is another possibility that deserves investigation if time and personnel permit.

Publications:

Potter, M., Wiener, F., and Mushinski, J.F.: Recent developments in plasmacytomagenesis in mice. In Klein, G. (Ed.): Advances in Viral Oncology 4. New York, Raven Press, 1984, pp. 139-162.

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Mountz, J.D., Mushinski, J.F., and Steinberg, A.D.: Differential gene expression in autoimmune mice. Survey of Immunological Res. 4: 48-64, 1985.

Yang, J.Q., Bauer, S.R., Mushinski, J.F., and Marcu, K.B.: Chromosome translocations clustered 5' of the murine c-myc gene qualitatively affect promoter usage: Implications for the site of normal c-myc regulation. EMBO J. (in press).

Erikson, J., Miller, D.A., Miller, O.J., Abcarian, P.W., Skurla, R.M., Mushinski, J.F., and Croce, C.M.: The c-myc oncogene is translocated to the involved chromosome 12 in mouse plasmacytomas. Proc. Natl. Acad. Sci. USA (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05553-16 LGN

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunoglobulin structure and diversity. Characterization of cell membrane proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.:	Stuart Rudikoff	Microbiologist	LGN,NCI
	E. Jouvin-Marche	Visiting Fellow	LGN,NCI
	A. Hartman	Staff Fellow	LGN,NCI
	W. Davidson	Visiting Associate	LGN,NCI
	R. Nordan	Staff Fellow	LGN,NCI

COOPERATING UNITS *(if any)*

N. Hansen, Assoc. Prof., Univ. of Md., College Park, MD

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.5	4.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

I. Gene evolution. Recombinant DNA libraries have been constructed from a variety of wild mouse species. These libraries have been screened with probes corresponding to the β chain of the T cell receptor, the immunoglobulin kappa light chain constant region, a kappa variable region gene family and a heavy chain variable region gene family. Sequence analysis of these genes from Mus pahari, the most distantly related mouse species to inbred animals, reveals different patterns of evolution and mutation for each of the genes studied. Thus, selective and evolutionary pressures appear to be acting quite differently on immunologically important structural genes.

II. Growth and regulation. A factor produced by the P388D1 macrophage-like cell line has been identified which is required by a number of plasmacytoma cell lines for growth in vitro. This factor is currently being purified and characterized and experiments are being initiated to assess its role in plasmacytomagenesis or normal growth.

PHS 6040 (Rev. 1/84) GPO 914-918

Objectives:

I. Mutational mechanisms in evolution. A series of genes whose products participate in defined, but different, immunological functions are being characterized in several wild mouse species to assess the mutational mechanisms acting on these genes in natural populations during a relatively short evolutionary period.

II. Control of growth and differentiation. A new program is being initiated in this laboratory to study the control and regulation of B cell growth and differentiation. This problem is being approached from two perspectives. The first is a characterization of factors which have been demonstrated to be involved in normal or neoplastic growth and/or differentiation. The second involves a study of genes directly involved in or regulating these processes through the use of subtractive DNA libraries generated from cloned B cell lines representing various stages of differentiation. These lines will be derived spontaneously or by induction from the same clonal progenitors.

III. Operation of protein microsequencing facility

Methods Employed:

Gene structures are determined by recombinant DNA technology involving construction of recombinant bacteriophage libraries, screening of these libraries with ^{32}P-labeled probes, followed by isolation of recombinants containing homologous sequences. These recombinants are then characterized by restriction mapping and appropriate fragments sequenced using the Sanger chain termination method.

Growth factors are being purified using a variety of physical-chemical techniques including size separation, reverse phase high pressure liquid chromatography and isoelectric focusing. Purification progress is monitored by both bio-assays and gel analysis.

A series of clonally related cell lines representing various stages of B cell differentiation are being established by growing pre-B cell lines in the presence of various B cell mitogens. These lines are then analyzed with a series of monoclonal antibodies recognizing antigenic determinants appearing at various stages of B-cell differentiation. Fluorescence activated cell sorting will be employed to isolate differentiating subpopulations for subsequent cloning and construction of cDNA libraries.

Major Findings:

I. A series of recombinant bacteriophage 'libraries' have been constructed from a number of wild mouse species representing a large spectrum of the evolution of the species Mus. These libraries have been screened with several probes corresponding to molecules playing important roles in the immune system including the β chain from the T cell receptor, the kappa light chain constant region, a kappa light chain variable region family, and a heavy chain variable region family.

To date (and with the support of NCI contract N01-CB-33934), six genomic libraries have been constructed and screened. The most extensive results have been obtained from M. pahari, the most evolutionary distant species from inbred mice in which most comparable studies have been performed. Genes corresponding to the β chain of the T cell receptor, the κ light chain constant region and five genes from a kappa light chain V region family (VK24) have been cloned and sequenced. A comparison of these genes between M. pahari and M. musculus domesticus (inbred mice) has provided interesting and important data on the mechanisms and processes by which genes evolve within a species.

An analysis of the β chain of the T cell receptor from M. pahari reveals surprisingly few substitutions. The β chain locus in inbred mice includes two β chain constant region genes, CTB1 and CTB2, which are extremely homologous in structure. This same locus structure is also found in humans, suggesting that the gene duplication giving rise to CTB1 and CTB2 occurred at a quite distant time in evolution.

Surprisingly, in a comparison of the large CTB exon I (375 bp) from an inbred mouse strain only a single substitution was found between CTB1 and CTB2. This result suggested that some type of correctional mechanism was operating to 'homogenize' the CTB sequences. Analysis of CTB genes from M. pahari reveals a total of 4 nucleotide differences between CTB1 and CTB2 only one of which causes an amino acid substitution. Furthermore, the M. pahari CTB genes were derived from a single recombinant clone so that they reside on the same chromosome and represent a single haplotype. Based on an assumption of random mutation, three times as many replacement substitutions (nucleotide substitutions resulting in amino acid replacements) would be expected than silent or non-coding substitutions. Clearly, the observation of 3 silent substitutions and one coding substitution is opposite to expected results. Also, based on generalized biological clocks an evolutionary divergence of approximately 1% amino acid substitution per 0.7 million years (myr) would be expected. The observed 0.8% amino acid difference between CTB1 and CTB2 would place their divergence at approximately 1 myr which clearly is not the case as homologous genes are found in inbred mice, which are believed to be separated by approximately 10 myr in evolution from M. pahari, as well as in humans. The number of nucleic acid substitutions between CTB1 (13) and CTB2 (14) from M. pahari and inbred mice is also considerably lower than would be expected from normal mutational mechanisms. These results suggest that a strong selection exists against substitutions and/or that a corrective mechanism, such as gene conversion, is operating to homogenize the CTB genes and prevent the generation of diversity.

In contrast to the unusual conservation observed in the CTB genes, the coding region of the kappa light chain constant region gene displays a pattern of evolutionary diversity much closer to predicted models. Twenty one substitutions (14 of which are replacement) are found between M. pahari and inbred mice. The ratio of replacement to silent substitutions, as well as the calculated species divergence of 9.2 myr based on these results is consistent with other data dating the evolutionary divergence of these species and predicted patterns of unselected gene evolution. However, when sequences of the κ chain enhancer region (a nucleotide sequence region approximately 600 bp 5' to the coding region which results in increased light chain expression) and the 5'

flanking region of the κ constant gene were examined the % mutation between M. pahari and inbred mice was found to be the same for all three regions. This result demonstrates that the enhancer region and, surprisingly the 5' flanking region are evolving at the same rate as the coding region. Thus, additional functional properties may be associated with the 5' region resulting in its marked conservation in comparison to 5' flanking regions of most other genes.

The third and most complex comparison made between these two species involves a kappa chain V region family consisting of 4-5 members. Three functional members of the VK24 family have been characterized in the BALB/c mouse and these genes are designated VK24, VK24A, and VK24B. Using the VK24 gene as a probe, a series of homologous clones were isolated and characterized from the M. pahari library. Analysis of these clones has permitted an examination of the evolution of a multi-gene family in contrast to the single copy C κ gene and the CTB1, CTB2 complex.

Two genes were sequenced which demonstrated 95% homology in the VK coding region to VK24. While these genes could potentially be alleles, Southern blot analysis has yet to reveal an individual with less than two hybridizing bands using the VK24 probe suggesting that these two sequences may alternatively represent recently duplicated non-allelic genes. Similarly, two genes were found which are 94% homologous to the BALB/c VK24A sequence. These two genes are non allelic based on distribution in individual animals and low sequence homology in parts of the 5' region. A single gene homologous to the BALB/c VK24B sequence was found and the coding region of this gene was the least similar of any pairwise comparison (87%). However, homology in the 5' region of VK24B was generally higher than the coding region in contrast to the other comparisons where coding regions were always more homologous than non-coding regions.

The pattern of evolution within a multigene family thus appears quite different than within pauci-gene families. Two instances are found (VK24 and VK24A) where possible gene duplications exist although this has not been rigorously proved in the case of VK24. Variation in patterns of conservation between sequences 5' to the coding region and the coding region sequences indicate that considerable variation exists in the mutation rate in various DNA segments 5' to the coding regions among similar genes. In the case of the VK24B genes, the higher homology in the 5' region could indicate recombination between the flanking region and a related V region although several other interpretations are equally feasible.

Our studies have shown that for each of the genes examined different patterns of evolution are observed. The T cell receptor β chain is unusually conserved and may be 'corrected' fairly frequently in evolution by mechanisms such as gene conversion. The coding segment of the kappa constant region gene is evolving in a manner 'typical' of structural genes although both the 5' enhancer and 5' flanking region genes show no more mutation than the coding sequence in contrast to most genes in which much higher variation is found in the 5' flanking region. The VK gene family exhibits a complex pattern of evolution with coding region homologies ranging from 87-95% and 5' flanking regions from 79-93%. The pattern

among the VK genes indicates that regions of the various gene segments appear to be under different selective and evolutionary pressures resulting in a variety of observed structures which cannot be readily placed in well defined categories. These results demonstrate the complexity of multi-gene family evolution and the problems inherent in attempting to define genetic mechanisms within such families.

II. Studies have been initiated to characterize and purify a factor produced by the macrophage-like cell line P388D1. This factor has been shown to be necessary for the in vitro growth and survival of a number of plasmacytoma cell lines and may be involved in vivo in either plasmacytomagenesis or normal lymphocyte growth. The factor has been initially subjected to size exclusion on porous beads followed by reverse phase high pressure liquid chromatography. These procedures have resulted in a recovery of approximately 50% activity in the context of 10 protein bands on silver stained gels. Additional procedures such as HPLC ion exchange chromatography and chromatofocusing are currently being examined as further purification steps prior to immunization of rats in an attempt to raise monoclonal antibodies to the factor.

In a second approach to the problem of control of growth and differentiation initial experiments have been performed aimed at the development of a B cell model which will include stable sublines derived from the same progenitor clone and representing various stages of differentiation. Pre-B cell lines have been grown in the presence of LPS for up to 30 days and the phenotypes of these lines assayed periodically by fluorescence activated cell sorter.

Initial studies indicate that several of these lines differentiate in vitro as judged by the expression of new antigens. Experiments are being initiated to clone out and characterize these new cell types for use in gene analysis of growth and differentiation.

III. Considerable effort has been spent in the last year on the operation of a protein microsequencing facility. Continuous problems have been encountered with the instrumentation and the feasibility of maintaining this facility is in doubt.

Significance to Biomedical Research and the Program of the Institute:

I. The findings described above on gene evolution have provided important data in understanding the mutational patterns and mechanisms operating on a variety of structurally important genes in natural populations. Since evolution of genes such as these is basic to the survival of individuals, our analysis of these processes is necessary to an understanding of the origin of genetic variance which in many instances has deleterious consequences.

II. The identification and purification of a growth factor necessary for plasmacytoma survival in vitro presents the opportunity to potentially assess the role of this molecule in in vivo neoplasia and/or normal growth.

Proposed Course of Research:

Work is continuing.

Control of Growth and Differentiation (Wendy Davidson)

Objectives

1. Control of B cell growth and differentiation

Two approaches are being taken to study the control of growth and differentiation of normal and neoplastic B cells. The first involves the study of the genes involved in the regulation of B cell differentiation and will make use of cDNA subtractive libraries prepared from B cells arrested at different stages of differentiation.

The second approach involves the study of B cell growth factors. Attempts will be made to biochemically characterize an autocrine growth factor produced by the NFS-1 series of B cell lymphomas.

2. T cell receptor gene expression in the abnormal subset of T cells that predominates in C3H mice homozygous for the genes lpr and gld

The autosomal recessive genes lpr and gld cause lymphoproliferative and autoimmune disease in mice. Although these two genes are non-allelic, they both cause severe lymphadenopathy that results from the outgrowth of a phenotypically and functionally abnormal subpopulation of T cells. These cells are Thy-1+, Ly-1+, L3T4-, Ly2-, sIg-, Ia- and also express the B cell antigen Ly-5 B220. They do not exhibit Ig gene rearrangements and are thought to be thymus-derived. To characterize these putative T cells further, it is proposed to prepare DNA and RNA from purified Ly5 B220+, Thy-1+ cells from C3H-lpr/lpr and C3H-gld/gld LN and from continuous T cell lines established from SJL-lpr/lpr LN and spleen to determine whether rearrangements of the T cell receptor β chain gene occur and whether mRNA species specific for the α, β and γ chains of the T cell receptor are produced.

Methods Employed

Pre-B cell lines for use in the preparation of subtractive B cell libraries were analyzed for cell surface markers on the fluorescence-activated cell sorter (FACS) using a panel of monoclonal antibodies. These cells were induced to differentiate in vitro by continuous co-cultivation with LPS for 1-4 weeks. Phenotypic changes indicative of differentiation were assessed by FACS analysis. Clonally related cell lines representing various stages of B cell differentiation will be established by single cell cloning using the EPICS cell sorter. The cDNA libraries will be prepared using the Okiyama-Berg technique or the λ gtll vector system.

A sensitive ^{3}H-TdR uptake bioassay has been developed for detecting the B cell growth factor produced by NFS-1 cells. The growth factor will be purified by using a variety of physical-chemical techniques, including size fractionation, HPLC chromatography, etc., that have been developed in the laboratory for this purpose.

Major Findings

I. To prepare a B cell cDNA subtractive library containing copies of transcripts of genes that regulate B cell differentiation, two B cell populations are required. First, a mature B cell capable of secreting Ig but not terminally differentiated into a plasma cell and second, a cell that can be identified on the basis of cell surface markers (e.g., Lyb2, Mac-1 and Ly5 B220) as a B cell that does not yet have its H or L chain Ig genes rearranged. Ideally, the pre-B cell would be capable of being induced to differentiate in vitro to a more mature B cell phenotype and to rearrange its H and L chain Ig genes.

Because cloned normal B cells at these two stages of differentiation are not available, we chose to use cloned B cell and pre-B cell lymphomas for the preparation of the library. Although cloned lines with a mature B cell phenotype are readily obtainable, few early pre-B cell lines have been described. The first task was to take a panel of pre-B cell lines and identify the most immature. Two previously studied pre-B cell lines NFS-5 and NFS-25 and 5 new lines, NFS-112, -467, -1135, -70 and HAFTL-3 were examined. Following stimulation with LPS for 1, 2 or 4 weeks the pre-B cell lines were tested on the FACS for the de novo expression of B cell markers. Although LPS stimulation did not induce mass differentiation of any of the pre-B cell lines, a small percentage of the cells (5-10%) acquired B cell surface markers (sIgM, kappa, Ia and ThB) associated with more mature B-cells. Following cloning these differentiated cells will be examined for Ig gene rearrangements and compared to their non-stimulated precursors which are currently being examined for rearrangements of IgH and L chain genes. Most of the unstimulated cell lines have at least one H chain gene rearranged while the L chain genes are in the germline configuration. Recently, another pre-B cell line, HAFTL-1, has become available which has the surface marker phenotype of a pre-B cell and has both H and L chain genes in the germline configuration. This line is being treated with LPS at present. If HAFTL-1 can be induced to differentiate it may be the pre-B cell of choice for the preparation of the library.

II. Attempts to clone the B cell lymphoma line NFS 1.2 were unsuccessful unless the cells were cultured in supernatants derived from high density cultures of NFS 1.2 or the related lines, NFS 1.0, NFS 1.1 and NFS 1.3. NFS 1.2, therefore, appeared to make an autocrine factor required for its own growth. To study this factor further at ^{3}H-T uptake assay was developed in which varying numbers of tumor cells are cultured with different concentrations of standard supernatant preparations. Studies are presently being undertaken to determine whether other B cell and pre-B cell lines also make this factor and whether it is produced by T cells. Supernatants from a panel of B cell, pre-B cell and T cell lines are also being tested to determine whether they produce a growth factor similar to that produced by P388D1 cells. If the factor produced by NFS 1.2 is found to be unique, attempts will be made to purify it and characterize it biochemically.

III. Six T cell lines were established from SJL-lpr/lpr LN and spleen and one line from C3H-gld/gld LN. DNA was prepared from these lines and from purified LN T cells from C3H gld/gld mice and examined for rearrangements of the β chain of the T cell receptor. Preliminary results showed that in each case only the germline configuration was observed. Work is now in progress to prepare RNA from the cell lines and DNA and RNA from Ly5 B220+ T cells from mice homozygous for lpr and gld. Northern and Southern blots will be prepared and hybridized with probes for the α , β and γ chains of the T cell receptor.

Significance to Biomedical Research and the Program of the Institute

The approach being used to study B cells at the molecular level will provide information on the mechanisms by which normal B cell growth and differentiation are controlled. This knowledge should be critical to ultimately understanding the events that lead to malignant transformation of B cells.

The nature of the T cell lesion caused by the autoimmune disease-inducing genes lpr and gld has not been elucidated. Further characterization of these unusual T cells at the molecular level may provide insights into their involvement in the disease process.

Proposed Course of Research

The projects outlined above were only recently initiated. Work is proceeding along the lines proposed.

Isolation and Characterization of Plasmacytoma Growth Factor
(Richard P. Nordan)

Objectives

In BALB/cAnPt mice, plasmacytomas (PCT) arise exclusively in the granulomatous tissue which forms in response to the intraperitoneal administration of pristane. This suggests that their growth is dependent upon microenvironmental influences provided by this inflammatory environment. In order to identify factors involved in this process, PCT cell lines have been established in this laboratory which are dependent upon one such factor, PCT-GF, for proliferation and survival in vitro. PCT-GF is produced by normal spleen cells peritoneal macrophages and the P388D1 macrophage cell line. The studies outlined here address the initial steps which are necessary to evaluate the role of PCT-GF in plasmacytomagenesis and possibly in normal immunological regulation. The ojectives of these studies have been to determine if any known lymphokines or other factors are responsible for PCT-GF activity and to characterize PCT-GF from biological, biochemical and molecular biological viewpoints. A number of purified factors have been obtained and screened for their ability to substitute for PCT-GF. We are proceeding with the isolation of PCT-GF to be used in experiments designed to determine a partial amino acid sequence, to clone the gene encoding PCT-GF and to produce antisera and monoclonal antibodies to PCT-GF.

Experiments to examine the effect of PCT-GF on cell cycle parameters and the expression of cell surface transferin receptors are underway in collaboration with Dr. Leonard Neckers. In addition, homogeneous PCT-GF will be used to determine if PCT cells possess PCT-GF receptors and to examine the distribution of the putative receptor on normal and malignant cell populations.

Indomethacin, an inhibitor of prostaglandin synthesis, inhibits pristane-induced plasmacytomagenesis. With the use of bioassays and antibodies specific for PCT-GF, we will evaluate the levels of PCT-GF produced by peritoneal cells during PCT induction and will determine if one effect of indomethacin is to reduce PCT-GF levels and if this is the result of an inhibition of PCT-GF production or a reduction of PCT-GF producing cells.

Major Findings

1. PCT-GF is distinct from other well characterized factors including IL-1, IL-2, IL-3, macrophage-CSF, BCGF (BSF-P1), EGF, TGFβ, and γ and βIFN, none of which are able to support the growth of the factor dependent PCT cell lines.

2. Pristane-primed adherent peritoneal cells (APCs) produce >50 fold higher levels of PCT-GF than do normal control APCs, suggesting a link between elevated levels of this factor and the induction of PCTs in BALB/cAnPt mice. PCT-GF activity is also produced by normal human peripheral blood monocytes. The production of PCT-GF by normal human and mouse cells suggests that PCT-GF may play a role in the growth and differentiation of normal (B?) cells.

3. P388D1-derived PCT-GF has been purified to a level of ten bands (on silver stained SDS-PAGE) with a 50% recovery of starting activity. PCT-GF activity comigrates on SDS-PAGE with one of the ten bands and has an apparent size of 23kDa. PCT-GF is a polypeptide with apparent isoelectric points of 6.2 and 6.4.

4. Collaborations with Dr. Leonard Neckers have revealed that in PCT-GF-dependent PCT cells, PCT-GF releases a block in the G1 phase of the cell cycle and positively regulates transferrin receptor (TFR) mRNA levels and membrane TFR expression. PCT-GF has no effect on the cell cycle parameters of PCT-GF-independent PCT cells. Such cells constitutively express high levels of membrane TFRs.

Methods

PCT-GF-dependent (T1165, T2027) and -independent cell lines (M460D) are cultured in vitro using P388D1 supernatant (SN) as a source of PCT-GF. A sensitive bioassay for PCT-GF has been developed which utilizes ^{3}H-TdR incorporation by the T1165 cell line. This assay is now highly mechanized and computer assisted data analysis has been developed and implemented. Various purified factors were obtained and screened for their ability to substitute for PCT-GF.

For the purification of PCT-GF, we are focusing on the use of high recovery techniques including controlled pore glass beads and reversed phase HPLC. In order to achieve homogeneity, an additional high-recovery chromotography step is now under evaluation. In order to obtain sufficient amounts of PCT-GF, optimized culture methods have been developed for the large scale production of high-PCT-GF-activity from P388D1 supernatant under serum-free conditions.

Specific rabbit antiserum and rat or hamster monoclonal antibodies to PCT-GF will be produced.

Significance to Biomedical Research and the Program of the Institute

The existence of a factor (PCT-GF) which is required by plasmacytoma cells for proliferation and survival raises the possibility that normal B cells require PCT-GF at some stage of differentiation and that one step in the transition to a malignant PCT is the constitutive response to this factor. The characterization of PCT-GF is an important step in dissecting the events which lead to the malignant phenotype and may also provide insights into B lymphocyte development.

Proposed Course of Research

1. In the laboratory of Dr. Stuart Rudikoff we plan to proceed with the isolation of PCT-GF, to determine a partial amino sequence of PCT-GF and to clone the gene encoding PCT-GF.

2. To produce monoclonal antibodies to examine the role of elevated PCT-GF levels in plasmacytomagenesis.

3. To determine if PCT-GF-like activity has a similar role in any human tumors (e.g., myeloma or Burkitt's lymphoma).

Publications:

Leonard, W.J., Depper, J.M., Crabtree, G.R., Rudikoff, S., Pumphrey, J., Robb, R.J., Kronke, M., Svetlik, P.B., Peffer, N.J., Waldman, T.A., and Greene, W.C.: Molecular cloning and expression of cDNAs for the human interleukin-2 receptor: Evidence for alternate mRNA splicing and the use of two polyadenylation sites. Nature 311: 626-631, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08726-08 LGN

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemistry and molecular biology of transplantation antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.:	Michael J. Rogers	Research Chemist	LGN,NCI
	Richard Swerdlow	Staff Fellow	LGN,NCI
	Giorgio Galetto	Visiting Associate	LGN,NCI
	David Siwarski	Bio. Lab. Tech.	LGN,NCI
	Louis Matis		
	Lloyd Law	Chief, Lab. of Cell Biology	LCBGY,NCI
	Gilbert Jay	Expert	LMV,NCI

COOPERATING UNITS *(if any)*

Dr. John Coligan, NIAID; Dr. Giorgio Parmiani, National Cancer Institute, Milan, Italy; Stuart Rudikoff, LGN,NCI

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this work is to investigate various biological and chemical properties of two types of murine cell surface antigens that induce graft rejection: histocompatibility antigens (H-2) and tumor associated transplantation antigens (TATA).

In the case of TATAs, the approach is to purify and characterize the molecules bearing these antigens from tumor cells. Polyclonal and monoclonal antibodies and specific T cell clones that recognize these molecules may then be prepared and used to investigate their biological properties. Ultimately, suitable DNA probes can be prepared and used to study the genes which encode the molecules. This structural information will lead to an understanding of the mechanism of induction of these antigens and their relationship to the oncogenic process. The structure of these molecules may also provide insights into some of the unique immunogenic properties of tumors, e.g., their ability to escape an apparently strong antitumor immune response.

In the case of H-2 antigens, the approach is to utilize alloantisera and monoclonal antibodies recognizing class I determinants to examine the molecules expressed on normal and neoplastic cells. Moreover, DNA probes and molecular cloning techniques can be used to study the organization and expression of the genes that encode the molecules. Current specific aims are to identify molecules coded for by the many class I genes present in the mouse genome and to obtain information about the evolutionary history of this polymorphic multigene family.

Project Description

A. Tumor Associated Transplantation Antigens

Objectives:

A central problem in tumor immunology is the identification of those cell surface molecules that are targets for the anti-tumor immune response. The purpose of this work is to identify, purify and characterize those molecules from one T-cell tumor, RBL-5. The tumor was chosen because it bears a very potent, easily solubilized tumor associated transplantation antigen, a necessary prerequisite for purification.

Major Findings and Proposed Course of Research:

Gp175 is a 175,000 dalton cell surface glycoprotein purified from RBL-5 cells, which is distinct from known viral or H-2 gene products. Immunization of C57BL/6 x BALB/c F_1 (CBF_1) mice with gp175 at doses < 1 µg mediates protection against challenge by RBL-5 or other cross-reacting tumors induced by these viruses. A similar protein is found on other tumor and normal cells that do not express a TATA cross-reacting with RBL-5. Since the protein purified from an unrelated cell cannot immunize mice against RBL-5 challenge, an alteration or mutation of the gp175 expressed on RBL-5 cells is evidently responsible for its immunogenicity. Our current goal is to identify this alteration.

Our strategy is to first localize the immunogenic epitope to the carbohydrate or protein portion of gp175 and then further characterize its structure. Initial attempts to use the tumor rejection assay for this purpose were unsuccessful because careful quantitative experiments are necessary and the *in vivo* assay is only semi-quantitative.

Since syngeneic TATA specific antibodies are also not available, we looked for immune T-cells specific for gp175 in collaboration with Dr. Louis Matis and Dr. Michael Bookman of the DCT. Antigen dependent T-cell clones were established from lymph nodes of immunized CBF_1 mice. These clones are MHC restricted, L3T4+, Ly2-, and are not cytotoxic for RBL-5. They do not respond to viral structural proteins or virus infected spleen cells. Proliferation specific for gp175 is observed at concentrations as low as 1ng/ml (10^{-12}M). The extreme sensitivity of these clones to electrophoretically purified gp175 demonstrates that this glycoprotein and not some minor contaminant is indeed the molecule bearing the TATA. Furthermore, these clones are 100-500 fold more sensitive to gp175 from RBL-5 cells than a similar protein from a tumor expressing a non-crossreacting TATA. Various chemical modifications of native gp175 produce responses from these cells that indicate they are recognizing polypeptide structure(s) of the molecule, and not carbohydrate moieties. Using these cells, we intend to isolate proteolytic peptides from gp175, and identify immunogenic peptides for sequencing. The peptide sequences from immunogenic and non-immunogenic forms of gp175 should help us understand the antigenicity

of this molecule. They will also provide a starting point for the preparation of a synthetic DNA probe to study the gene encoding this interesting protein. In other work related to the TATA project, we have shown that RBL-5 contains additional TATA bearing molecules besides gp175 and, in fact, may contain many such molecules (Giorgio Galetto, et al., submitted to Int. J. Cancer). This has led us to suggest that purification by conventional biochemical procedures yields the most chemically stable TATA bearing molecule but not necessarily the most immunogenic. In order to test this hypothesis we are utilizing three tumor systems where TATA bearing molecules have been purified to homogeneity. Antisera against these molecules will be used to quantitatively remove them from a whole cell lysate. We will then check to see how much of the total cellular TATA remains. Preliminary results indicate that these purified molecules represent only a small fraction of all of the immunogenic cellular structures.

Based on these latter studies we have concluded that purification of individual tumor antigens is not the best way to investigate the molecular nature of TATAs. In collaboration with L. Matis and M. Bookman we have embarked upon a new procedure where mice are immunized with RBL-5 tumor cells and helper or cytotoxic T-cell lines are established by stimulation with total glycoprotein fractions from RBL-5. In this way, the T-cells recognizing the most immunogenic protein can hopefully be selected. These T-cells will be tested for in vivo anti-tumor activity. Clonal analysis of these lines will provide information on the total number of TATA bearing molecules. The most abundant clones, presumably directed against the strongest antigen, may then be used to establish in vitro assays to further purify the TATA bearing molecule. In this regard, experience with anti gp175 clones indicates that SDS-denaturation does not completely destroy the immunogenicity of TATA bearing proteins. Hence, purification techniques involving denaturing conditions should permit rapid purification of TATA bearing molecules.

B. Histocompatibility Antigens

Objectives:

The objectives are: 1. To characterize the MHC derived molecules expressed on normal and tumor cells and correlate them with the MHC genes which encode them. This will help develop an understanding of the genetic mechanisms controlling the expression of these genes. 2. To isolate and characterize the MHC genes from wild mice distantly related to inbred strains in order to obtain information on the evolutionary history of a multigene family. By comparing sequences of the same gene from species of mice that have been evolving independently for millions of years, alternative routes of evolution from a probable common ancestor may be compared. This will provide information on the mechanisms maintaining the polymorphism of this family of genes. Moreover, since highly conserved DNA sequences within distantly related genes may be inferred to have functional significance, this approach may reveal previously undetected functional sequences.

Major Findings and Proposed Course of Research:

In our continuing efforts to study the expression of histocompatibility antigens on tumor cells, we had shown that the SJL/J tumor, DMLM 1678, expressed two additional class I molecules besides the usual H-2K^s and H-2D^s molecules. One, encoded by genes in the D region, is expressed in very low quantities and is also expressed on normal lymphocytes. The other, encoded by a gene mapping to the K region, is present in about one half the amount of the major K region molecule and is also found on normal SJL/J lymphocytes.

The second K region molecule was identified by virtue of its slightly lower molecular weight that could not be attributed to glycosylation or phosphorylation. In collaboration with Dr. John Coligan's group, we have now established that the two H-2K region encoded molecules differ by tryptic peptide mapping. However, they have the same 22 residue amino terminal sequence and the same C-terminus as judged by reactivity with an anti-C terminal serum. Thus, the difference between the two molecules lies somewhere in the central portion of the polypeptide. The results suggest that the two molecules may arise from heretofore unknown alternative splicing events at the mRNA level and we are currently investigating this idea.

2. Using a DNA probe for class I genes and Southern blotting techniques we recently confirmed the observation of Lorraine Flaherty that BALB/cJ and BALB/cAn substrains contain a polymorphism associated with the Qa2 gene. We are currently utilizing this polymorphism to investigate the possibility that a BALB/cJ tumor underwent fusion with host cells during passage in BALB/cAn mice. If true, this would provide evidence that tumors passaged in syngeneic mice or growing in the original host may exchange genetic information with normal host cells.

3. Our studies on the evolution of class I genes in wild mice have significantly advanced over the past year. We had shown that the class I genes of mice distantly related to inbred mice were highly homologous in both coding and noncoding regions and obtained evidence that the number of class I genes can vary significantly.

We have now shown in collaboration with Gilbert Jay's group, that one gene, Q10, which encodes a secreted class I molecule, is conserved in distantly related species and it appears that this gene can be uniquely distinguished from the multitude of other class I genes by a single copy DNA probe. Thus, the opportunity exists to examine the same class I gene from a number of species representing alternative routes of evolution.

We have now cloned several class I genes hybridizing to the single copy Q10 probe from a genomic library constructed from liver DNA of the species Mus pahari, a remote ancestor of the inbred laboratory mouse Mus musculus domesticus. So far, we have sequenced two of these genes from just 5' of exon

4 through the 3' noncoding region and into the 3' flanking region. Several preliminary conclusions may be drawn, although further sequencing is necessary to fully appreciate the relationships of these class I genes to those of inbred mice. First, neither gene could encode a secreted molecule and, therefore, the sequences unique to the Q10 gene identified by the single copy probe may also be associated with other class I genes in this species. Second, the 3' noncoding regions of these genes contain repetitive alu sequences very similar to those found associated with the H-2D^d and H-2L^d genes of inbred mice. Third, some regions of the gene are highly homologous to class I genes of inbred mice, others are not. These data suggest that parts of various class I genes may be shuffled during evolutionary time and that different segments of the same gene are under different selective pressures.

We are continuing our search for the Mus pahari analogue of the Q10 gene from inbred mice and plan to extend this search to other species. We have also recently begun a collaboration with Dr. L. Flaherty to look for the Q6 gene in these mice with a single copy probe that she is developing. This gene appears to encode the Qa2 molecule.

Significance to Biomedical Research and the Program of the Institute:

In spite of the fact that mammalian hosts develop a readily detectable immune response against most tumors, the tumor usually grows progressively. While many models have been proposed to explain this phenomenon, none have been conclusively proven. The principal reason for this is that the molecular target of the immune response is, in most cases, totally unknown. Without knowledge of the target, definitive experiments to test the various models are not possible. Thus, our attempts to purify a tumor associated transplantation antigen are extremely important to the understanding of the host response to tumor growth and to the development of rational immunotherapy protocols.

The histocompatibility antigens are of crucial importance for immune recognition and graft rejection and related molecules are apparently involved in hematopoietic differentiation. These functions are of obvious significance to tumor immunology and leukemogenesis. Important unresolved issues are the physiological functions of class I proteins encoded by the many class I genes besides H-2K and H-2D and how the extreme genetic polymorphism of these molecules relates to their presumed functions. Our research program attempts to address these basic biological questions.

Publications:

Siwarski, D.F., Prat, M., and Rogers, M.J.: A novel H-2^s class I molecule expressed on a B-cell leukemia from SJL/J mice. Mol. Immunol., in press.

Galetto, G. and Rogers, M.J.: The molecular nature of multiple tumor associated antigens expressed by a Rauscher-MuLV induced leukemia. In Franks L.M. (ed.): Cancer Surveys, in press.

Rogers, M.J., Germain, R.N., Hare, J., Long, E. and Singer, D.S.: Comparison of MHC genes among distantly related members of the genus Mus. J. Immunol. 134: 630-636, 1985.

Siwarski, D.F., Barra, Y., Jay, G., and Rogers, M.J.: Occurrence of a unique MHC class I gene in distantly related members of the genus Mus. Immunogenetics 21: 267-276, 1985.

Rogers, M.J., Galetto, G., Matthai, R., and Potter, M.: Qa2 expression in BALB/c sublines and a BALB/cLAC tumor. In Potter, M. (ed.) Current Topics in Microbiology and Immunology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05552-16 LGN

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mammalian cellular genetics and cell culture

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

P.I.:	H. G. Coon	Research Biologist	LGN,NCI
	R. DiLauro	Visiting Scientist	LB,NCI

COOPERATING UNITS *(if any)*

Dr. F. Saverio Ambesi-Impiombato, Istituto di Patologia Generale, Naples, Italy

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It is the purpose of this project to analyze and develop new and difficult systems for cell culture. Four related but separate problems are now active in our laboratory. I. For 30 years the usual goal of in vitro cell biology has been to fragment the more complex tissues and organs by developing clonally purified cell strains. There has been considerable success in isolating differentiated cell strains. We are now trying to reverse this process and to reconstruct more complex tissues and organs from well characterized, cloned cell strains from the rat thyroid gland: follicle cells, c-cells, fibroblasts and parathyroid cells. These cell combinations have been tested as grafts in thyroidectomized animals. II. In a collaborative effort we are examining the expression of the thyroglobulin gene by testing in a transient expression transfection assay various constructs from the 5' sequences of a cloned rat thyroglobulin gene in differentiated follicle cells (FRTL-5) and in mutant derivatives of these and other thyroid-derived cell strains. III. We are attempting to culture the elements of and to reconstruct in vitro functional olfactory epithelium from rats. The sensory neurons of the olfactory epithelium are renewed throughout life from a stem cell population. Development of this system would make available the first mammalian neuroblast to neuron cell culture system. It is hoped that basic issues in olfactory sensory physiology can be explored with this system. IV. The germinal cells of the testis in the F1 generation from a BALB/c x Czech mouse cross are arrested at prophase I of meiosis. Such arrested cells can be isolated in quantity and divide in culture and as heterokaryons with dividing somatic cells.

Project Description

I. Thyroid gland reconstruction

Objectives:

We have attempted to assess the functional capacities (the normality) of cloned rat thyroid cell strains (FRTL, FRTL-5) that have remained remarkably differentiated in culture for many years. Our tentative conclusions are that these cells are functionally normal based on tests of subcutaneous grafts into thyroidectomized animals. We want to go ahead and try to look at the effect of including other clonally purified cells from the thyroid gland in the grafts. We would like to find conditions permitting more rapid weight gain and better health in the grafted animals as well as gaining some insight about the interaction of specific cells in the thyroid gland.

Methods Employed:

We have chosen to reimplant FRTL and FRTL-5 cells into young rats that have stopped growing after thyroidectomy, hoping to show that these cells, as grafts, can supply T3/T4 and allow resumption of weight gain, thereby mimicing the action of the normal thyroid gland. We shall include cloned, functional parathyroid cells, c-cells, thyroid endothelial cells and fibroblasts in various combinations in these grafts.

Major Findings:

We have found that in order to get timely vascularization these normal (diploid, differentiated, non-tumorigenic) cells must be included in a collagen gel and apparently accompanied by fibroblasts (from skin or thyroid). The FRTL cells have retained the ability to form hollow, spherical follicle-like structures and to establish intimate relations with the host capillaries. To date, four animals that received one million or more cells in the graft resumed weight gain within a month or two after grafting. In at least one case it has been ascertained that no "rests" or ectopic thyroid tissue was found in the throat tissues. The others remain to be checked but no evidence of ectopic thyroid activity has been found in animals that have failed to resume weight gain for eight weeks after thyroidectomy. It appears that T3/T4 production was due to the action of the grafted assemblies.

Significance to Biomedical Research and the Program of the Institute:

While the thyroid gland does not have to be replaced after removal (T3 and T4 replacement therapy is simple and effective), there are other endocrine functions (e.g., the parathyroid) and other tissues and organs for which the possibility of replacement may be expected to benefit from the development of this technology. Already skin grafts and artificial small caliber blood vessels have been made from collagen assemblies like those used here. It is important to note that these experiments offer important evidence against the widely held belief that cells age in cell culture or they become transformed and

tumorigenic, usually losing their differentiated functions in the process. The FRTL cells used in these experiments have undergone more than 350 population doublings in culture and still are normal in karyology and are appropriately functional both in vitro and in vivo. It is increasingly important that cell systems that can be treated (e.g., transfected) or merely propagated (e.g., skin) in vitro be developed for experimentation and evaluated for their ability to be reimplanted in host animals. The longstanding question of tumorigenic hazard after culture may be evaluated in a realistic way using these methods. We believe that it is important to learn about the interactions of characterized cell strains in the in vivo environment.

Proposed Course of Research:

We plan to build on the beginning that we have made by increasing the number of cells in the grafts and to test the effect of adding other thyroid-derived cells. We expect to fool around with the composition of the collagen matrix used in the grafting by including some of the basement membrane matrix materials available from George Martin (NIDR).

II. Tissue specific expression of thyroglobulin gene - collaboration with Dr. Roberto DiLauro (LB, NCI) and Dr. Saverio Ambesi (Naples, Italy)

Objectives:

Dr. DiLauro's group in Naples, Italy, has been working for the past three years at cloning the gene for thyroglobulin (TG) from rats. They have isolated the entire gene and sequenced the majority of the gene. We are interested in the regulation of this gene. Because we have the differentiated FRTL cell strains as well as other thyroid-derived, non-TG-producing cell strains we are able to examine the tissue specific expression of the TG gene. This is done by inserting putative regulatory sequences of the native TG gene in association with easily recognized markers. The goal of this work is to identify first the portion(s) of the cloned sequence that are needed for tissue specific expression and, secondly, to find what molecule(s) interacts with that sequence.

Methods Employed:

The 5' sequences (about 1500 bp) from the TG gene were ligated to the chloramphenicol acetyl transferase (CAT) gene and these sequences tested by using a transient expression, transfection assay using $CaPO_4$. The vector's promoter was also tested without any TG 5' sequences. FRTL-5 thyroid cells and BRL-30E rat liver cells as well as a standard mouse 3T3 as control. Two to four days later the populations that had been exposed to the DNA-$CaPO_4$ coprecipitate were tested with radioactive chloramphenicol. Autoradiographs of chromatograms made from cell extracts were used to detect acetylated chloramphenicol.

Major Findings:

To date the major finding has been that tissue-specific expression of the CAT gene does occur; thus far, only the differentiated, FRTL-5 thyroid cells produced acetylated chloramphenicol when the TG 5' sequences were present, but all cell types produced it when the same constructs minus the TG 5' region were transfected. Recent experiments have narrowed the apparent tissue-specificity conferring region to some 125 bp just 5' to the start of the coding region.

Significance to Biomedical Research and the Program of the Institute:

These experiments, which have thus far been done almost entirely by Dr. DiLauro and his associates in Maxine Singer's lab, point up the important role of the cell culture systems that have been developed in our lab (we have supplied the cell strains used in these studies and will be involved in the selection of stable transfections which are to be made next). Without the differentiated in vitro cell systems and their attendant variants and mutants one would have to use transgenic mice (an experiment which is contemplated nevertheless) in order to test tissue specific gene expression. These experiments, like others of their kind involving other genes, may be expected to yield information crucial to an ultimate understanding of gene regulation, the developmental processes that are involved bringing about that regulation, and prospects and techniques for therapeutic intervention.

Proposed Course of Research:

Much work remains to be done before these results are ready for formal publication. Other regions in introns and 3' to the coding sequences may also have an important effect. We want to study stable transfectants and their behavior in different cell strains. We anticipate the testing of this regulatory region in transgenic mice.

III. Biology of the Olfactory Epithelium

Objectives:

The mammalian olfactory epithelium (OE) represents a timely challenge to cell biologists, neurobiologists and cell culturists. The cellular mechanism for the chemoreception in the OE is not understood. Presumably, there are receptors in the dendritic tree of the sensory cells, but how many types of receptors? how many receptors per cell? A particularly intriguing feature is that these sensory neurons have half lives of 30 days in mouse and man and are, therefore, constantly being renewed from a stem cell population. The organ is planar, a pseudostratified epithelium, and therefore well suiited for bringing into cell culture, where goals would be to clone and compare receptor activity among clones. The development of cell strains with characteristic responses to different oderants would be a great advance in the attempt to understand both neuroblasts in general and to understanding olfactory receptor biology in

particular. The history of past attempts implies that it will be crucial to success to culture the whole system of OE cells (at least 3 cell types) in order to find conditions for normal functions to be expressed in vitro. The epithelium should also be studied in situ or after the cribriform plate and proximal turbinates are removed (from neonatal rat pups). These preparations could be examined using voltage sensitive fluorescent dyes in the hope of identifying differential patterns of activity in cells from various regions and how these patterns might change during challenge by different oderants.

Methods Employed:

Three new culture methodologies combine to make it seem likely that culture of the OE might be achieved soon. a) The key cell is the basal or stem cell. Like other stem cells it is characterized by keeping a foot always planted on a basement membrane. George Martin (NIDR) has available basement membrane gels that have proven valuable in culturing cells from sources difficult to culture. b) Our new found insights into the serum-free culture methods for neurons, and certain constants about growth factor requirements of such cultures hold new promise for success. c) The technique which I call serial co-culture may very well prove almost uniquely well suited to the OE challenge: first, make cultures as usual and accept whatever cell type grows or can be maintained; then, using this as a feeder layer, add fresh primary cells until the entire tissue can be maintained with each cell type represented.

Major Findings:

We have mastered the dissections and the tissue dissociation methods. Satisfactory cultures have not yet been obtained.

Significance to Biomedical Research and the Program of the Institute:

As noted above there is no cell level knowledge of the physiology of the sensory cells of the OE. The OE is almost virgin territory. Successful culture of these cells promises a meaningful advance in the study of (neuro)blast → neuron differentiation as well as various intriguing aspects of sensory coding in a system that may show specific mapping in a way similar to the retinotectal system. The mystery inherent in the challenge of recognizing as many as 10,000 different oderants poses unique problems for receptor biology perhaps not unlike those posed for the immune system a decade ago.

IV. Cell fusion studies of mouse germinal cells arrested in prophase I of meiosis

Objectives:

The physiological differences between mitotic and meiotic cells remain obscure. We found quite by accident that in the testes of infertile mice in the F1

generation of a laboratory cross between BALB/c and Czech I (Mus musculus musculus) many cells were arrested in prophase I of meiosis in late diakinesis. There were no more mature forms, metaphase cells or spermatids to be found. These testes provide an excellent source of prophase I cells for experimentation. The initial goals of this experimentation are to discover whether the arrested cells will enter metaphase and mitosis in the cytoplasm of a cell that does undergo mitosis and to look for reciprocal evidences for influences of the special characteristics of each type of division upon the other. We are interested in looking for possible haploid derivatives of such hybrid divisions.

Methods Employed:

We have dissociated the seminiferous tubules from the testes of affected F1 mice and fused these in suspension and on established monolayers to cells that have selectable markers using inactivated Sendai virus. Heterokaryons formed are examined over the next few days by a variety of standard methodologies: fixation and staining (for metaphase counts), selection in HAT medium for hybrid cell formation and using antibodies to differentiated products (thyroglobulin) normally found in the cytoplasm of the non testis parent cell in some heterokaryons.

Major Findings:

We have found that the arrested meiotic prophases are able to complete a division in the cytoplasm of a normal somatic cell. From the dramatically increased number of anaphase bridges seen in such cells we conclude that the meiotic cells have undergone recombination and are segregating parental chromatids as in a reductional division. The block that is effective in the testes is reversed in the cytoplasm of a somatic cell. Both mouse and rat somatic cells have given this result. Somatic cell hybrids were formed and some of these showed drastic reduction in chromosome number from the model number typical of the selectable parental line. These hybrid cells cannot yet be interpreted.

Significance to Biomedical Research and the Program of the Institute:

The fundamental similarities and differences between meiotic and mitotic cells are truly basic to all higher cell systems. We hope that experiments like this can add to that presently too small fund of information. The behavior of germinal cells whether teratocarcinomas or normal germinal cells has long been believed to be fundamental to an understanding of processes like development and differentiation.

Publications:

Bell, E., Moore, H., Mitchie, C., Sher, S., and Coon, H.: Reconstruction of a thyroid gland equivalent from cells and matrix materials. J. Exp. Zool. 232: 277-285, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08950-03 LGN

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunochemistry and genetics of protein-binding immunoglobulins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Sandra Smith-Gill	Sr. Staff Fellow	LGN,NCI
	C. Mainhart	Microbiologist	LGN,NCI
	T. B. Lavoie	Biologist	LGN,NCI

COOPERATING UNITS *(if any)*

W. Drohan, Molecular Genetics Group, Meloy Laboratories, VA; K. Dorrington, Dept. of Biochemistry, University of Toronto, Toronto, Canada; D. Davies, LMB, NIADKD; D. Benjamin, Dept. of Microbiology, Univ. Virginia, Charlottesville, VA

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Monoclonal antibodies directed against model protein antigens are used as probes to study antibody-protein interactions and structure-function relationships, and to study developmentally regulated antigens in normal and neoplastic development. In order to define the complementary structure of an antibody and a protein epitope as precisely as possible, antigenic regions and specific epitopes recognized by monoclonal antibodies to three well characterized proteins, lysozyme c, ovomucoid, and staphylcoccyl nuclease, are examined. Epitopes are mapped by comparing antibody reactivity with related proteins, and results to date have revealed significant relationships between antigenic and tertiary structure. The antibodies are analyzed structurally by sequencing, chain recombination studies, crystallography and computer modelling, and results to date reveal some important properties of the antibody combining site in an anti-protein immunoglobulin which may differ significantly from those of anti-hapten immunoglobulins. Structurally and functionally related antibodies are compared to determine genetic mechanisms underlying anti-protein specificity. Experiments are in progress to generate monoclonal antibodies to onc gene protein products; these antibodies will be used to study these proteins in normal and neoplastic B-cell development.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description

Objectives:

The long term objective of this project is to utilize monoclonal antibodies as specific probes to study the structure and function of developmentally regulated proteins in normal and neoplastic cells. The immediate specific goals are two-fold: 1) to develop model systems of antibody-protein interactions, using well characterized model protein antigens, in order to define the relationship between antigenic structure and the tertiary structure and functional characteristics of the protein antigen; and 2) to develop monoclonal antibodies to several biologically important proteins, to use these antibodies to purify and characterize structure-function relationships to these proteins, and to examine the expression of these proteins in plasmacytoma cells and in normal morphogenesis using immunochemical methods.

Methods Employed:

Monoclonal antibodies (mAbs) specific for three well defined proteins (hen egg white lysozyme, ovomucoid, and staphylcoccal nuclease) are being investigated as model systems for defining the protein-protein interactions underlying antibody recognition of a protein antigen. The approach includes several facets. First, mapping the epitopes recognized by the mAbs allows definition of the antigenic structure of defined proteins as well as functional grouping of mAbs based upon binding specificities. These results provide a model for interpretation of studies utilizing mAbs to probe the antigenic structure and structure-function relationships of biologically important proteins whose structure and function are less well defined.

Second, in order to understand the structural basis of antibody specificity for a protein, the complete sequences are determined for selected mAbs, and their 3-dimensional structure determined through model building, and ultimately by protein crystallography. Structure-function relationships can then be inferred by comparison of closely related antibodies and experimentally examined first by chain recombination experiments, and then by in vitro expression of both the antigen and antibody, and site-specific mutagenesis to test hypothesized relationships.

Over 100 mAb each to hen egg white lysozyme (HEL) and to ovomucoid have been prepared by hybridoma technology. The staphylcoccal nuclease (SN) system is being developed in collaboration with Dr. D. Benjamin, University of Virginia, who has prepared a panel of mAb specific for SN which was obtained in a bacterial expression vector. The epitope recognized by each monoclonal antibody is mapped by comparing the reactivity of each antibody with a battery of sequenced proteins from various species, and by comparing the patterns of competition among the antibodies for binding to the protein antigens.

The V_L and V_H genes expressed in each mAb were initially characterized by N-terminal sequencing of the proteins; current and future studies will utilize RNA dot blotting and RNA sequencing methods to survey and group structurally

related mAbs. In order to define the complementary structure of an antibody and a protein epitope as precisely as possible, the primary structure of the V regions of the heavy and light chains of selected antibodies have been determined initially by a combination of protein sequencing, in collaboration with Dr. S. Rudikoff, and by cloning and sequencing the cDNA from anti-lysozyme hybridomas in collaboration with Dr. W. Drohan, Meloy Laboratories. Current sequencing studies are concentrating on sequencing of cDNA clones and of mRNA by specific primer extension, in collaboration with Dr. K. Huppi.

The three-dimensional secondary and tertiary structures of the sequenced proteins are being defined, both through computer modelling and emergy minimization in collaboration with M. Potter and C. Mainhart, and through crystallography. In collaboration with Drs. D. Davies and E. Silverton (LMB, NIADKD), the Fab portions of HyHEL-8, -9, and -10 have been prepared and crystallized individually and in complex with the antigen, lysozyme. The HyHEL-10 complex is currently under study at 3Å resolution. With the three-dimensional model of the antibody determined, and the three-dimensional structure of the protein epitope similarly constructed from known coordinates of HEL, the interaction of the antibody with the specific epitope has been modelled, and will ultimately be refined by protein crystallography studies.

In order to examine the relationships between antibody structure and function, chain recombination experiments among anti-HEL antibodies and a variety of non HEL-binding antibodies have been performed in collaboration with Dr. K. Dorrington, Toronto University, to determine functional correlates of the various structural variations among closely related heavy and light chains, as well as the kinetics of heavy and light chain pairing. The rearranged light and heavy chain genes have been cloned from HyHEL-10 and HyHEL-8 in collaboration with Drs. W. Drohan and G. Ricca, Meloy Laboratories. These have been sequenced to allow comparison of the sequences of the expressed genes with the germline genes in order to assess the amount of somatic mutation. These genes are being utilized for in vitro expression and selective modification by genetic splicing mechanisms to directly test hypotheses concerning structure-function relationships.

In order to generate monoclonal antibodies to be used as probes for the c-myb and c-myc oncogene protein products, mice and rats have been immunized with synthetic peptides corresponding to the amino acid sequences predicted from v-onc and/or c-onc gene sequences. Hybridoma technology has been used to produce mAb specific for the peptides.

In addition, attempts to express segments of cloned cDNA clones in bacterial vectors are underway in collaboration with Dr. J.F. Mushinski. We hope to obtain larger peptide fragments for use in immunization. Any mAb or polyclonal antisera obtained are then to be tested for ability to immunoprecipitate v-onc or c-onc protein from cell lines known to be producing mRNA for these proteins. Possible clones will be selected and used to develop immunochemical and immunohistochemical assays for the onc products to be studied during normal and neoplastic B-cell development.

Major Findings:

The studies using defined model protein antigens have yielded several important conclusions. Although the antigenic structure of a protein is very complex, a number of properties may be defined. (1) The entire surface of a protein is probably antigenic, and the number of unique epitopes is very large. (2) Interactions among individual mAb with the protein antigen and with each other is closely related to known tertiary structure of the antigen. Based on patterns of competitive and of simultaneous binding to HEL, the mAbs were divisible into 3 complementation groups that operationally define antigenic regions which correspond to structural domains of HEL. The domain localization of epitopes appears to be the most important factor in determining interactions among mAb: mAbs recognizing the same domain cannot simultaneously cobind and always reciprocally compete, while interactions among mAbs recognizing distinct domains may be complex, including nonreciprocal interactions and enhanced binding. The interactions among mAbs binding a single domain are currently being investigated utilizing those mAbs specific for the isolated third domain of ovomucoid. (3) Most mAb recognize determinants which include either bends in a β-sheet, or a break in an α-helix, i.e., a "corner" in a polypeptide chain which protrudes and exposes backbone atoms to potential interactions with the antibody. These regions would correlate with regions of high "mobility" on the protein surface.

The complete V_L and V_H sequences of the mAb HyHEL-10 were determined and physical models of HEL and the mAb HyHEL-10 were constructed. The HEL model was constructed from published x-ray coordinates. The V_L and V_H regions of HyHEL-10 were very short, and HyHEL-10 was constructed by deleting the long loop segments of L1, H2 and H3, and joining the open ends, but without otherwise changing the energy minimized torsional angles of McPC603.

Several notable features are evident from the model of HyHEL-10: (1) the antibody combining region is a very shallow concavity approximately 18 x 25 Å , a direct consequence of the short lengths of L1, H2 and H3 (11, 16 and 8 residues, respectively); (2) the concavity is acidic and nonhydrophobic, and is bordered by hydrophobic segments; (3) a prominent feature of the 3-dimensional structure, not immediately evident from the primary structure, is the cluster of tyrosine residues over the L3 and H2 areas; these tyrosines are contributed primarily by H2.

The epitopes most likely recognized by HyHEL-10 and by the related mAb HyHEL-8 have been localized to domain I of HEL. It includes a segment containing residues 18-27 that are located in a bend between 2 alpha helices on HEL domain I and forms a surface of approximately 17 x 24 Å .

The most structurally compatible fit between HEL and the HyHEL-10 model was between the proposed epitope on HEL domain I and the H3 concavity of HyHEL-10. The dimensions of concavity closely approximate those of the epitope. The primary CDRs in contact with HEL are L2, H1 and H3. When the 2 molecular models are juxtaposed, there is an extremely close complementarity between the backbone conformations and the physical properties (charge and hydrophobicity) of the individual residues in contact on the opposing surfaces. In addition, there is a high complementarity of side chains with the backbones:

of at least 27 potential hydrogen bonds readily identifiable between the 2 molecules, the majority involve backbone molecules, and of these 16 are between the side chains of one molecule and the backbone residues of the other. These results suggest that side chain-to-backbone hydrogen bonding may be an important component of specificity in this type of complex. The functional predictions generated by this model are currently being tested by further chain recombination experiments and by site specific mutagenesis on expressed V_H and V_L genes. The model itself will ultimately be tested from x-ray analysis of crystallized Fab and Fab-HEL complex.

In chain recombination experiments with the antibody proteins, reconstituted anti-HEL mAbs showed relative affinity andd fine specificity patterns comparable to those of the native parental molecules. Recombinant molecules among anti-HEL and anti-hapten mAbs showed that the ability to bind HEL vs. galactan or DNP is absolutely heavy chain determined, but structurally unrelated L-chains can often support relatively high affinity H-chain binding to HEL. Recombinant molecules were made between J539, a galactan-binding myeloma protein which expressed V_HX-24 and V_K4, and HyHEL-5, a mAb specific for an epitope including Arg68 which expresses V_HJ558 and V_K4. The results demonstrated that both the J539 and HyHEL-5 L-chains are capable of binding galactan but not HEL when recombined with the appropriate heavy chains. Similarly, recombinant molecules among 3 mAbs all expressing V_H36-60 and V_K23, HyHEL-8 and -10, and XRPC-25, a DNP-binding myeloma protein, demonstrated that specificity for DNP vs. HEL is also heavy chain determined. Sequence data suggests that differences in H3 are responsible for DNP vs. HEL specificity in these mAbs.

With respect to relative affinity for HEL or DNP, and domain localization of binding on HEL, the L-chains of HyHEL-8, -10, and XRPC-25 appeared equivalent. However, fine specificity analyses revealed important L chain contributions and demonstrated that for the 2 mAb HyHEL-8 and -10 fine specificity is a cooperative feature of the 2 chains. For example, HyHEL-10 is 40 fold more sensitive to the japanese quail egg white lysozyme (JQEL) substitutions than HyHEL-8. A similar relationship holds for the reconstituted HyHEL-10 and HyHEL-8 molecules as judged by competitive inhibition ELISA. However, when chimeric molecules are tested (H10-L8 or H8-L10), the level of inhibition is intermediate to the parent molecules. Part of the structure that is responsible for HyHEL-10's greater JQEL sensitivity is present in the V_H region and part in the V_L region. Analysis of the V_L rearrangement patterns using Southern blot analysis syggests that the two antibodies utilize the same V_K germ line, thus light chain-determined fine specificity differences can be attributed to somatic mutation.

Recombinant molecules among anti-HEL mAbs specific for different (nonoverlapping) epitopes revealed that the domain localization of binding was frequently but not always heavy chain correlated. Important exceptions included recombinant molecules in which HyHEL-8 or -10 L chains predominated specificity. Another exception included recombinants with HyHEL-5, which suggested that an unusual deletion in the V-J joint site in the HyHEL-5 L3 is important to recognition of the HyHEL-5 epitope. These results as well as recombinants among closely related anti-HEL mAbs specific for closely related (overlapping) epitopes, demonstrated that both domain specificity and fine specificity results

from an interaction of heavy and light chains. In some cases, fine specificity differences may be attributed to only a few specific amino acid differences among mAbs.

In summary, several important conclusions have emerged with respect to structure-function relationships in protein-binding antibodies: (1) the antibody response to a given protein is structurally very heterogeneous with a great diversity of V_L-V_H expression; (2) the ability to bind the protein antigen HEL, versus several haptens, is absolutely heavy chain determined; (3) the fine specificity, (e.g., specific epitope recognized on the protein surface) is frequently heavychain predominated, but involves a complex heavy and light chain interaction; (4) the antibody combining site may be very large, involving a much greater surface area than for hapten-binding, and the shape may not be of the typical "groove" or "cavity" conformation proposed for haptens, but rather more of an open face with a shape complementary to the structural domains of the protein antigen and complementarity-determining-regions protruding into hydrophobic pockets between the structural domains; (5) somatic diversification mechanisms (V-J and V-D-J joining, and somatic mutation) may be the primary determinants of anti-protein specificity, in contrast to haptens where V_L-V_H pairing appears to predominate.

To date, a total of 18 fusions have been performed utilizing mouse or rat spleen cells hyperimmunized in vivo and/or in vitro with carrier-conjugated v-myc, c-myc or c-myb synthetic peptides. Hybridoma clones secreting mAb for the peptide have been isolated from each fusion. Growth of many of these clones has been very poor, perhaps due to cross-reactivity of the mAb with the endogenous proteins. In those cases where antibody was successfully isolated, they proved to be of the IgM class and of low affinity and specificity. These results are consistent with several recent publications which document that the peptide-induced mAbs have very low affinity and specificity for the native protein. We tested several of our mAbs which proved negative for ability to immunoprecipitate any protein from an appropriate tumor. Current efforts are concentrating on obtaining protein from cloned genes expressed in vitro, in hopes that they will prove to be better immunogens.

Significance to Biomedical Research and the Program of the Institute:

An understanding of the nature of antibody-protein interactions is of both basic and applied significance. Protein antigens, especially those of viral and tumor origin, probably represent a primary source of selective pressure on the immune system, but to date our knowledge of the structural basis of anti-protein antibody specificity is limited. The results to date of this project have yielded several important new conclusions which contribute to our understanding of the molecular basis of antibody diversity and specificity, and which will ultimately contribute to our ability to "design" antibodies with predetermined specificity through genetic engineering. On the other hand, our experience with the synthetic peptides underscores the necessity and importance of a detailed understanding of antigenicity and immunogenicity, especially in light of curent interest in but limited success with "synthetic vaccines." In addition, a detailed knowledge of the nature of antibody interactions with defined, model proteins

provides a necessary basis for interpreting results when mAb are used as probes for expression and function of biologically important proteins, including oncogene products, whose structures are essentially unknown. Understanding the role of these proteins in normal and neoplastic development is essential to understanding the molecular steps in cellular transformation.

Proposed Course of Research:

Increasing emphasis is being placed on study of proteins which may be important in the development of neoplasia, including the oncogenes myb and myc, which are in progress. Studies on the model protein systems will concentrate detailed interactions of several well-defined antibodies, a structural and functional comparison of antibodies induced to peptides with those induced by the native protein, and on the role of somatic mutation in the evolution of the immune response.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08951-03 LGN

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular basis for the acute erythroleukemias induced by murine retroviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.:	S. K. Ruscetti	Senior Investigator	LGN,NCI
	L. Wolff	Sr. Staff Fellow	LGN,NCI
	S. W. Chung	Visiting Fellow	LGN,NCI

COOPERATING UNITS *(if any)*

Dr. M. Potter, LGN,NCI; Drs. W. F. Anderson, P. Kantoff and D. Trauber, Lab. of Molecular Hematology, NHLBI

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies have been aimed at trying to understand mechanisms by which murine leukemia viruses induce erythroid transformation and to understand why some strains of mice are resistant to one or more of the stages of the malignant process.

Investigations on the acute erythroleukemia-inducing virus, spleen focus-forming virus (SFFV), have been several-fold. Comparisons of the molecular structure of two variants, $SFFV_P$ and $SFFV_A$, and formation of a recombinant virus have allowed localization of a region within the envelope gene that determines the ability of infected cells to differentiate in response to erythropoietin. Other experiments have shown that the long terminal repeat (LTR) region of SFFV, unlike Friend murine leukemia virus, does not carry sequences required for tissue-specific induction of leukemia, since a number of different LTRs can be substituted without affecting the disease latency or phenotype. And finally, the generation of helper-free SFFVs using a packaging cell line has provided proof that helper virus is not required for in vitro transformation of erythroid cells, first stage acute erythroleukemia in mice or development in vivo of second stage tumorigenic cells.

Studies on the genetics of susceptibility to early erythroleukemia induced by Friend murine leukemia virus have identified a gene on chromosome 5, at or near the Rmcf locus, that plays a major role in resistance of mice to this disease by preventing the replication of mink cell focus-inducing (MCF) viruses, believed to be the proximal cause of the disease. This gene is believed to be either a structural gene or a regulatory gene for an MCF virus-related envelope glycoprotein that appears to block the cell surface receptor for MCF viruses. Additional genes, acting through unknown mechanisms, may also be involved in resistance.

Major Findings:

(1) The vast majority of the genetic differences between two variants of the spleen focus-forming virus reside in the 3' half of the envelope gene.

Two variants of the spleen focus-forming virus (SFFV) induce acute erythroleukemia in mice but differ in their effects on erythroid cells. Cells infected with $SFFV_A$ retain their dependence upon erythropoietin (Epo) for differentiation, whereas cells infected with $SFFV_P$ appear to proliferate and differentiate without the need for Epo. Both viruses encode a 52,000 dalton glycoprotein, gp52, but only the $SFFV_P$ protein is post-translationally modified to a 65K form that can be detected on the cell surface. Previous studies with molecular clones of these viruses have localized their biological effects to a region containing the envelope (env) gene and the long terminal repeat (LTR) sequences. In order to identify DNA sequences within these genes potentially responsible for the biological and biochemical differences between $SFFV_P$ and $SFFV_A$, the env gene of $SFFV_A$ was sequenced and compared with the previously published sequence of $SFFV_P$. The LTR regions of both viruses were also sequenced. The results indicate that the nucleotide differences in the env genes between $SFFV_P$ and $SFFV_A$ result in 32/377 animo acid changes that are located mainly in the carboxyl end of gp52. When the LTRs were compared, only 6 differences were noted out of the 517 base pairs sequenced, 5 of which were in the U3 region and 1 in the R region. These results indicates that the vast majority of the genetic differences between $SFFV_P$ and $SFFV_A$ reside in the 3' half of the env gene.

(2) A recombinant virus between $SFFV_P$ and $SFFV_A$ localizes the biological and biochemical differences between the two viruses to a region encompassing the 3'end of the env gene.

In order to assess the role of the 3' half of the env gene in determining the biological and biochemical differences observed between $SFFV_P$ and $SFFV_A$, a recombinant virus was prepared which contains the 3' half of the $SFFV_P$ env gene and the $SFFV_P$ LTR on an $SFFV_A$ background. Analysis of this recombinant indicates that it is capable of inducing all of the biological effects previously associated with $SFFV_P$. Mice infected with this virus develop grossly enlarged spleens within 21 days, have elevated numbers of reticulocytes and a great increase in the number of erythroid colonies in the spleen (CFU-E) that are capable of differentiating in vitro in the absence of erythropoietin. In vitro infection of bone marrow cells with this virus leads after 5 days to a large number of hemoglobinized erythroid bursts in the absence of erythropoietin. Spleen cells from mice infected with this recombinant virus do not require Epo for proliferation and do not proliferate in response to Epo, a property associated with $SFFV_P$ but not $SFFV_A$. Thus, substitution of 3' env gene sequences in $SFFV_A$ with the analogous sequences in $SFFV_P$ results in a virus that has all of the biological effects of $SFFV_P$.

Since we had previously observed that there were differences in the post-translational modification of the envelope proteins of $SFFV_P$ and $SFFV_A$, we were

interested in whether the env gene products of the recombinant virus were processed like $SFFV_P$ or $SFFV_A$. The results indicate that the recombinant virus containing the 3' end of the env gene from $SFFV_P$ is post-translationally modified to a 65K form which can be detected on the cell surface, a property associated only with $SFFV_P$.

The results obtained in this study strengthen the argument that the $SFFV_P$ env gene product is acting at the cell surface to affect the response of erythroid cells to erythropoietin since the 3' half of the SFFV env gene, which determines the hormonal requirements, contains sequences encoding the hydrophobic p15E-related transmembrane portion of the envelope glycoprotein.

(3) There are no differences in pathogenicity or target cell specificity of SFFV when the LTR region is replaced with LTRs from a variety of other MuLVs.

Previous studies on the LTR regions of ecotropic Friend MuLV (F-MuLV) have shown that it contains sequences, not found in Moloney MuLV (M-MuLV), which are capable of determining the erythroid nature of F-MuLV disease in newborn mice. We were interested in whether the particular LTR sequences present in SFFV, or the related F-MuLV or Friend mink cell focus-inducing (Fr-MCF) viruses, were required for pathogenicity and/or erythroid target cell specificity or whether they could be substituted for by the analogous sequences in the lymphoid leukemia-inducing Moloney MuLV or Moloney MCF virus.

Recombinant SFFVs were prepared using the 5' LTR, gag-pol, and env genes from SFFV in combination with 3' LTRs from F-MuLV, F-MCF, M-MuLV and M-MCF virus. The resulting viruses were pseudotyped with various helper viruses and tested in vitro and in vivo for erythroid cell specificity and pathogenicity. The results indicate that all of the recombinant viruses are capable of producing equivalent in vitro transformation of erythroid cells as well as inducing acute erythroleukemia in mice with all of the characteristics previously associated with SFFV-induced disease. These results, in combination with previous studies on the env gene of SFFV, indicate that the primary genetic determinants within SFFV responsible for inducing its characteristic erythroid transformation are found within the envelope gene of the virus.

(4) Helper virus is not required for multiple stages of SFFV-induced erythroid cell transformation.

Since SFFV is replication defective, it requires the presence of helper virus in order to induce an acute erythroleukemia. In order to determine if helper virus is essential for this leukemogneic process, we prepared helper-free $SFFV_P$ and $SFFV_A$ using ψ-2 cells, which contain a mutant Moloney MuLV genome that is missing the sequences required for packaging of its own RNA into viral particles. When these cells are transfected with SFFV DNA, they produce retroviral particles containing only SFFV RNA, since NIH 3T3 cells infected with culture fluids from these cells express SFFV, but not MuLV, envelope proteins and do not release reverse transcriptase. These helper-free SFFV

particles were then tested for their effects both in vitro, in an erythroid transformation assay, and in vivo, by direct intravenous inoculation of mice. When bone marrow cells were incubated with culture fluid from the SFFV-transfected ψ -2 cells and observed for colony formation in semi-solid medium, erythroid bursts could be detected after 5 days in culture. Those infected with helper-free $SFFV_A$ required the addition of exogenous erythropoietin, while those infected with $SFFV_P$ did not, consistent with previously reported results obtained using helper-containing stocks of virus. No erythroid bursts were observed on day 5 after incubation of bone marrow cells with fulture fluids from untransfected ψ -2 cells.

To assess the in vivo oncogenic potential of the helper-free SFFVs, mice were injected intravenously with culture fluids from SFFV-transfected ψ -2 cells. Some mice were pre-treated with phenylhydrazine to increase the number of target cells available to the virus, and this was found to be essesntial for demonstrating biological activity. Greater than 50% of adult NIH Swiss mice pre-treated with phenylhydrazine and then given helper-free $SFFV_P$ developed grossly enlarged spleens (as high as 1.94 grams in weight) 12-27 days post-innoculation. Cells from these spleens were indistinguishable histologically from those of mice given helper-containing stocks of $SFFV_P$, and they expressed high levels of the SFFV envelope glycoprotein, gp52. Injection of culture fluid from ψ -2 cells transfected with $SFFV_A$ into mice induced foci of transformed erythroid cells on the spleen surface at 9 days but was not able to induce a progressive disease unless it was concentrated 50-fold. In the latter case, it induced disease in about half of the mice. We were unable to find any evidence of replicating helper virus in the diseased mice infected with either helper-free $SFFV_P$ or $SFFV_A$. Spleens from these mice showed no evidence of helper-virus-encoded proteins and did not release reverse transcriptase, and cell-free homogenates from these spleens were unable to transfer replicating virus to NIH 3T3 cells or to induce disease when injected into other mice. These results indicate that (1) expression of helper virus genes in erythroid target cells is not necessary in conjunction with SFFV for the transformation process and (2) systemic infection which allows continued introduction of SFFV into new target cells is not required for fulminating disease.

Previous studies have shown that erythroid cells transformed by SFFV, in the presence of helper virus, can undergo multiple stages of malignancy giving rise to immortalized erythroid cells that are able to grow indefinitely either in vivo or in vitro. In order to determine if helper virus is required for conversion of first stage cells to cells having a more autonomous or malignant phenotype, we tested the tumorigenicity of erythroid cell populations transformed by helper-free SFFV. The results indicate that spleens enlarged as a result of helper-free SFFV transformation will give rise after several weeks to cells which can be transplanted serially into the omentum of syngeneic sub-lethally irradiated recipients. The autonomous nature of the cells derived from such a spleen could also be demonstrated in a quantitative in vitro colony assay for tumorigenic cells and by the ability of these cells to grow indefinitely as erythroleukemia cell lines. The latter cells, like their helper virus-containing counterparts, are inducible for hemoglobin synthesis with a variety of chemicals.

The fact that such cell lines are free of helper virus gene products attests to the oncogenic potential of the SFFV genome.

These studies with helper-free SFFVs show in a more general sense that non-replicating retroviral vectors containing transforming genes can be utilized to study the oncogenic effects of these genes, and that under conditions where target cells are accessible in vivo, it is possible to introduce a vector containing such a gene into animals by direct inoculation.

(5) Spleen cells from $SFFV_P$- and $SFFV_A$- infected mice proliferate in the presence of antibodies to erythropoietin.

Using a simple microassay based upon ^3H-thymidine incorporation into DNA, we previously observed that spleen cells from mice infected with $SFFV_P$ or $SFFV_A$ were able to proliferate to high levels in the absence of added erythropoietin, which was not true of spleen cells from normal, phenylhydrazine-treated or F-MuLV-infected mice. Although the addition of erythropoietin to the cultures has no effect on the level of proliferation of cells from $SFFV_P$-infected mice, it greatly increases the level of proliferation of cells from $SFFV_A$- and F-MuLV-infected mice, indicating that at least the latter cells retain their responsiveness to Epo. In order to rule out the possibility that $SFFV_P$- and $SFFV_A$-infected cells had acquired a hypersensitivity to Epo that allowed them to proliferate in response to low amounts of Epo that might be present in the fetal calf serum used in the assay, we carried out the assay in the presence of a high titered antiserum to Epo. An amount of antiserum sufficient to neutralize 200 mU of Epo had no effect on the level of proliferation of $SFFV_P$- or $SFFV_A$-infected cells in the absence of Epo, indicating that they were not proliferating in response to low levels of Epo in the serum. This same amount of antiserum reduced to background levels the proliferation of spleen cells from F-MuLV-infected mice in the presence of Epo, consistent with previous data that these cells maintain an absolute requirement for Epo for proliferation. The results with SFFV indicate that these viruses are able to alter a cell's requirement for a normal regulator of proliferation.

(6) Resistance of DBA/2 mice to acute erythroleukemia induced by F-MuLV can be mapped to the $Rmcf^r$ locus on chromosome 5.

Utilizing a series of BALB/c mice congenic for various DBA/2 genes, we were able to establish that DBA/2 mice carry a gene on chromosome 5, at or near the $Rmcf^r$ locus, that plays a major role in resistance to early F-MuLV-induced erythroleukemia. The fact that this gene controls the replication of MCF viruses strengthens the case for these viruses playing a crucial role in the disease, since failure to replicate MCF viruses results in resistance to early erythroleukemia. The data with the congenic mice as well as with (BALB/c x DBA/2) F_1 hybrids backcrossed to BALB/c mice also indicates that additional genes on other chromosomes may contribute to resistance to F-MuLV-induced early erythroleukemia by mechanisms other than blocking the replication of MCF viruses.

We had previously shown that DBA/2 mice constitutively express on the cell surface an MCF virus-related envelope glycoprotein that apparently blocks the receptor for MCF viruses, preventing their spread. Since the expression of the $Rmcf^r$ gene is correlated with the expression of this cell surface protein, it is likely that the $Rmcf^r$ gene is either a structural gene for this unique protein that can block the receptor for MCF viruses or is a regulatory gene that controls its expression. The Rmcf gene has no effect on the late myeloid, lymphoid or erythroid diseases that appear in DBA/2 and other strains of mice, consistent with data indicating that MCF viruses are not required for the development of these late diseases.

Significance to Biomedical Research

Studies with acute erythroleukemia-inducing viruses of the mouse, such as SFFV, have clearly shown that viruses can effect the proliferation and differentiation of hematopoietic cells without carrying or inducing an onc gene. The primary effect of these viruses on hematopoietic cells appears to be due primarily to the product of their env genes, which like onc genes may have counterparts in normal cell DNA. Later, these cells can develop into ones with a more malignant phenotype, making these virus-induced erythroleukemias good models to study tumor progression. Determining the mechanisms by which viruses such as SFFV cause changes in the proliferation and differentiation of hematopoietic cells and malignancy may give us insights into the cause and the treatment of similar diseases in man. Also, the study of the mechanisms of resistance to virus-induced leukemia in the mouse may also have relevance for the treatment of human leukemia.

Proposed Course of Research:

(1) Studies to further define the pathogenic region(s) of the SFFV envelope gene:

Nucleotide sequencing data has indicated that all strains of SFFV contain three major changes from their ecotropic parent, F-MuLV: an MCF gp70-related 5' end, a large deletion spanning the gp70/15E cleavage site, and a smaller and unique 3' end. Studies will be undertaken to assess the importance of each of these changes in pathogenicity.

(2) Further studies to determine the basis for the phenotypic differences between $SFFV_P$ and $SFFV_A$:

The recombinant virus between $SFFV_P$ and $SFFV_A$ that we have been studying contains LTR sequences as well as env gene sequences from $SFFV_P$. Although it is doubtful that any of the 6 nucleotide differences in the LTR region between $SFFV_P$ and $SFFV_A$ are responsible for the biological and biochemical differences observed between the two viruses, we are currently attempting to make a recombinant virus that contains only the env gene sequences from $SFFV_P$. Also, we will attempt to further localize the sequences within the 3' half of the envelope gene responsible for the differential effects that the two variant

viruses have on erythoid cells.

(3) Studies to determine the mechanisms by which the spleen focus-forming and Friend MCF viruses alter erythropoiesis:

Erythroid cells normally require erythropoietin for proliferation and differentiation, and infection with SFFV apparently alters this requirement. Since our molecular studies continue to indicate that the envelope protein of SFFV, gp52, is the crucial element for biological activity, further studies are being planned to understand how this protein modifies erythropoiesis. Our studies have shown no evidence that the viral envelope protein is related to erythropoietin or its receptor. Studies will be carried out to determine if the SFFV envelope protein is related to other growth factors or receptors involved in erythroid cell proliferation and differentiation. In addition, studies will be attempted to determine if SFFV is enabling erythroid cells to bypass the need for hormone/receptor interaction by directly providing or inducing the second signal for proliferation and differentiation.

Similar studies will be carried out to determine how the envelope protein of Friend MCF virus is involved in the apparent block in erythroid differentiation occurring in F-MuLV-infected mice.

(4) Further studies utilizing helper-free SFFV

The helper-free induction of SFFV-induced erythroleukemia provides an excellent model system for multistep malignant transformation. An advantage of this system is that progression of cells through stages of transformation is uncomplicated by the presence of infectious retroviruses which can continuously initiate new transforming events. Further experiments will look at mechanisms by which transformed cells that are not tumorigenic are transformed to a more autonomous, highly malignant phenotype. Initial studies will look at retroviral integration sites as well as rearrangements and increased expression of known onc genes. Since initial work has indicated that some inbred mice develop highly malignant cells more readily than others, further studies will be carried out on a variety of mouse strains and congenic mice to determine if resistance or susceptibility can be localized to a specific chromosomal region.

The experiments performed this year on erythroleukemia induction by a non-replicating vector carrying SFFV have tested a system which may have wide application in gene transfer studies. Experiments are being planned whereby various onc genes, either singly or in combination, can be transferred to cells in vitro or directly in vivo to assess their roles in the multistep malignant transformation of primary cells.

(5) Studies on the resistance of mice to F-MuLV-induced erythroleukemia:

Our studies with congenic mice have established that DBA/2, but not BALB/c, mice carry a gene on chromosome 5 at or near the Rmcf locus that plays a major role in resistance to early F-MuLV-induced erythroleukemia, and that expression

of this gene is associated with the expression of an MCF virus-related envelope glycoprotein on the cell surface that appears to block the receptor for MCF viruses. Future studies will focus on cloning the $Rmcf^r$ gene in order to determine whether it represents a structural gene for this unique envelope glycoprotein or a regulatory locus that controls expression of this *env* gene, and to determine whether introduction of this gene into cells from susceptible mice will prevent the replication of MCF viruses and the development of F-MuLV-induced early erythroleukemia.

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Annual Report Summary
Laboratory of Molecular Biology
DCBD, NCI
October 1, 1984 to September 30, 1985

Research in the Laboratory of Molecular Biology focuses on understanding the factors that control gene expression in animal cells and bacterial cells. This information is used to define the biochemical basis for the abnormal growth and behavior of cancer cells. In addition, there is a strong emphasis on investigating the role of the plasma membrane in receiving signals from hormones, growth factors, the extracellular matrix, and other cells and on determining how these signals are transmitted to the genetic apparatus. This information is used to devise new ways of cancer diagnosis and treatment.

Cancer Treatment:

Using the information previously gathered on the pathway of receptor mediated endocytosis I. Pastan, M. C. Willingham and D. Fitzgerald have tried to develop immunotoxins to treat human cancer. The toxin chosen was Pseudomonas toxin (PE) which has several advantages over other toxins. It is very active, very stable, and when conjugated to antibodies has very low toxicity on normal human cells. In collaboration with Dr. Thomas Waldman (Metabolism Branch), PE has been coupled to an antibody to the Interleukin 2 receptor (anti-Tac). PE-anti-Tac will kill cells from adult T-cell leukemia patients at 20 ng/ml. Thus it is a very effective killing agent for these cells. It is much less active against normal cells. PE-anti-Tac has been administered to monkeys and levels of 2 µg/ml have been obtained without significant toxicity to these monkeys. PE-anti-Tac was still present at significant levels in monkey blood 24 hours after administration. These studies indicate that PE-anti-Tac might be of use in treating adult T-cell leukemia patients. Approval for such studies has been obtained from the FDA and are comtemplated in the near future. (The study was done in collaboration with T. Waldmann.) Pseudomonas toxin has also been coupled to antibodies that recognize antigens present on ovarian cancer cells. Some of these agents have very high activity in tissue culture and their efficacy is being explored in a collaborative study with Dr. Robert Ozols, Medicine Branch, DCT, NCI, and scientist at Cetus Corporation.

Immunotoxins enter cells in endocytic vesicles termed receptosomes or endosomes, and must escape from this vesicular system into the cytosol in order to kill cells. Escape is very inefficient so that only a small fraction of the molecules internalyzed reach the cyotsol; most molecules are transferred to lysosomes where they are destroyed. To increase the efficiency of penetration of immunotoxin molecules into the cytosol two approaches have been taken. One is to find drugs that will enhance this process. Verapamil, a calcium channel blocker has this property. Verapamil analogs are being tested to see if some of these might be more potent enough to use in vivo. A second approach is to lyse or disrupt the membranes of endocytic vesicles. Adenovirus enters cells in endocytic vesicles and disrupts the membrane of the vesicle releasing the contents of the vesicle into the cytosol. We previously showed enhancement of immunotoxin action by adenovirus, biochemical studies have now been carried out to determine the basis of this action. It has been found that the three surface proteins of adenovirus, fiber, penton base and hexon undergo a pH dependent change in their properties. At pH 5 to 6, the proteins become hydrophobic and

bind non-ionic detergent tightly. This finding helps explain how adenovirus penetrates the membrane of the endocytic vesicle as follows: the endocytic vesicle is acidic and when the virus reaches this environment, its proteins expose hydrophobic residues that penetrate the membrane of the vesicle. The vesicle appears to be under osmotic tension and this high osmotic pressure probably causes rupture of the vesicle at the point of adenovirus insertion.

One of the obstacles to successful cancer chemotheraphy is the development of drug resistance and particularly, multidrug resistance. To determine the biochemical and genetic basis of multiple drug resistance, a human epithelioid cancer cell line (KB) has been mutagenized and used to select clones displaying high level multidrug resistance. Either colchicine, adriamycin or vinblastine were used as selecting drugs. Highly resistant cell lines contain amplified genes which code for the drug resistance. A revertant cell line which has lost multiple drug resistance has lost the amplified sequences. A DNA probe has been obtained by cloning the amplified sequences. This probe recognizes a messenger RNA of approximately 4.5 kilobases in length. This RNA is overproduced in multidrug resistant cell lines and probably codes for a protein having a role in multidrug resistance. This DNA probe is currently being used to study human tumors and human cancer cell lines to determine if the findings in cell culture are relevant to human cancers in patients (M. M. Gottesman and I. Pastan).

Internalized ligands are delivered into the cell together in the same vesicles, but may then separate to either recycle to the cell surface or be delivered to lysosomes by efficient mechanisms. Their segragation occurs in an elaborate, but ill-defined, array of membranous elements of the "trans" domain of the cell, elements that are connected functionally with the trans face of the Golgi stacks, as well as the plasma membrane. The Ultrastructural Cytochemistry Section has made an attempt to define morphologically the elements of this "trans" domain of the cell using specific morphologic cytochemical markers. These include sialic acid, specific lectin, as well as monoclonal antibodies to: a lysosomal membrane protein (LAMP-1), a lysosomal matrix (MEP), a Golgi stack protein (mc ABL-70), and two recycling receptors (alpha2-macroglobulin and transferrin receptors). A transferrin-ferritin conjugate and antibody to VSV "G" protein were used to compare a recycling ligand marker and a marker of exocytosis from the Golgi stacks. These studies have suggested a clear delineation between lysosomal and recycling pathways, but indirect functional connections between both of these and the Golgi stack system. The presence of the elements involved in recycling to the plasma membrane have been found in close proximity to those of the constitutive exocytic system, but direct membrane fusion of these elements has not yet been clearly found. These studies help to define the organelle locations in which segragation of proteins and ligands occur to aid in the understanding of the underlying biochemical mechanisms that might be responsible for these events.

Previous morphologic studies have implicated the coated pits of the Golgi region as having a role in intracellular sorting of ligands entering the cell by endocytic vesicles and eventually rooted to lysosomes. To determine the role of the coated pits of the Golgi in this process, a new biochemical has been developed. The coated membranes in the form of vesicles are isolated from homogenates by immunoadsorption onto Staphaureus coated with anti-clathrin antibody. Using ^{125}I- EGF, the transit of EGF could be sequentially followed, first through the coated pits of the cell surface and after an eclipse, through the coated pits of the Golgi on the way to lysosomes. We have also applied this method to study receptor entry by labeling the EGF receptor (or the transferrin receptor) with

methionine. Then EGF is added to the cells and homogenates made at frequent intervals. Coated vesicles are isolated from homogenates and their content of receptor determined by antibody precipitation. It was found that EGF drives the EGF receptor first through coated pits at the surface and later through coated pits of the Golgi. The transferrin receptor, which is not directed to lysosomes by transferrin, is only found in the first, plasma membrane associated, coated compartment. These studies strongly support our hypothesis that the clathrin-coated membranes of the Golgi have an important role in directing the EGF receptor, and probably other receptors to lysosomes by concentrating them out of the Golgi compartment (M.C. Willingham and I. Pastan).

The Molecular Cell Genetics Section (M.M. Gottesman) continued the genetic analysis of cultured somatic cells to determine the role of cAMP in regulation of cell growth and morphology. The involvement of tubulin in spindle formation and the mechanism whereby human cancer cells develop simultaneously resistance to multiple drugs. Dominant mutations conferring resistance to cAMP-mediated growth inhibtion have been transferred by DNA-mediated gene transfer to sensitive recipient cells. This assay is being used to clone the gene encoding a mutant regulatory subunit of type I cAMP dependent protein kinase. Similarly, a mutant β-tubulin gene conferring colcemid-dependence and a specific spindle defect has been transferred and amplified in recipient cells. Several independent multidrug resistant human KB carcinoma cell lines have been developed. These lines express specific amplified DNA sequences at levels at least 100-fold higher then the parent cell line.

Several studies on the mechanism of regulation of gene expression are in progress (G. Johnson) The role of ADP-ribosylation of nuclear proteins in mediating the genetic regulation effects of steroid hormones has been examined through the use of inhibitors of ADP-ribosylation and nuclear run-off experiments to measure transcription of MMTV sequences. The major excreted protein (MEP) of transformed mouse fibroblasts, a novel and activatable lysosomal protease, is also regulated at the level of transcription by the tumor promoter, TPA, and the growth factor, PDGF.

K. Yamada and members of the Membrane Biochemistry Section are analyzing the functions of adhesive glycoproteins and their receptors. Structural comparisons between species by S. Akiyama indicated that the two major forms of fibronectin are derived from a single gene. An evolutionarily conserved region containing the sequence Gly-Arg-Gly-Asp-Ser was shown to be a critical, specific recognition signal for fibronectin-mediated cell adhesion and movement in vitro, and collaborative experiments showed that such fibronectin peptides inhibited two major migratory processes in embryonic development-gastrulation and neural crest cell migration.

The binding of fibronectin to cells was shown by S. Akiyama to occur via a moderate-affinity receptor, which was directly inhibited by the conserved pentapeptide. A putative receptor for fibronectin was characterized by T. Hasegawa collaborating with W.T. Chen. It was a complex of 3 glycoproteins that partially co-localized with extracellular fibronectin and α-actinin inside cells. Monoclonal antibodies to this protein blocked cell adhesion to fibronectin, yet mimicked fibronectin adhesiveness if directly adsorbed to substrates. Collagen receptor function was analyzed by K. Nagata and others, and was shown to modulate fibronectin receptor function at specific steps, suggesting close relationships

between these receptors for 2 major extracellular proteins. Studies in progress as characterizing the mechanisms of action of the putative fibronectin and collagen receptors, and testing the efficacy of synthetic peptides in inhibiting a variety of adhesive and migratory events, including platelet function and tumor cell invasion and metastasis.

The Gene Regulation Section (B. de Crombrugghe) continues to study collagen regulation. Fibroblasts which have undergone malignant transformation show an important transcriptional inhibition of the type I collagen genes and other transformation sensitive genes. To understand the mechanisms which cause this inhibition in v-mos transformed NIH 3T3 fibroblasts, we have designed a selection to obtain mutants in which the expression of these genes is restored. The mutants are pleiotropic and contain a mutation probably in a cellular transacting factor which either directly or indirectly controls the expression of the type I collagen and fibronectin genes in v-mos transformed cells. Isolation of the mutated gene(s) is in progress. This novel genetic approach should help dissect the pathways of cellular reactions which are triggered by the oncogenic proteins and which cause the alterations in growth control and other aspects of the transformation phenotype of these cells.

Transgenic mice strains have been generated in which a collagen promoter fused to a marker gene, has been stably integrated in the germline of these mice. These mice exhibit stage specific and tissue specific expression of the chimeric gene which parallels the expression of the endogenous type I collagen genes.

The prokaryotic research groups in the Laboratory of Molecular Biology have been interested in molecular mechanisms of control of gene expression, proteolytic control mechanisms in cell and in DNA synthesis.

Sankar Adhya and his colleagues have continued studies of regulation of gal operon, rho gene and the molecular basis of cyclic AMP action in gene transcription in E. coli. They have shown that gal repressor binding to the two operators of the gal operon plays a more dynamic role in modulating transcription than was formerly envisioned. Instead of repressor sterically blocking RNA polyermase entry to promoter, two molecules of repressor bound to the two operators change the conformation of the promoter DNA structure making the latter inadequate for transcription initiation.

Cyclic AMP complexes with its receptor protein, CRP and the cyclic AMP. CRP complex binds in a sequence specific way to DNA to modulate gene expression. CRP is a two domain protein. Cyclic AMP binding to the amino domain induces an allosteric change, which imports specific DNA binding property to the carboxy domain. By isolating and studying mutants of CRP, which modulates gene without cyclic AMP, Susan Garges and Sankar Adhya have precisely defined the amino acids and thus the α-helicles which interact specifically to cause the allosteric shift. These studies also suggest that Vander Waal's repulsions between the side chains of these amino acids may be responsible for the intramolecular structural rearrangement during cyclic AMP induce allosteric change in CRP.
The interactions of multiple factors to regulate gene expression, DNA replication and recombination are being studied in E. coli and bacteriophage lambda. The powerful genetic approaches available in these systems allow components of these complex processes to be identified, which can then be analyzed in vitro. Genetic identification and cloning of three genes responsible for regulation of capsular polysaccharide synthesis suggests that one of the

regulatory proteins may be sensitive to proteolysis by the Lon proteolysis system. We have begun to study the in vitro specificity of Lon proteolysis, using as a substrate lambda N protein, which we have previously demonstrated to be an in vivo substrate for the system. The mechanism of N action for anti-termination of transcription in bacteriophage lambda is being investigated by mutational analysis of a host gene, in which suppressors of nusA mutants had been isolated. Mutations isolated on a cloned copy of the gene are being introduced into the host chromosome to define the role of this protein for E. coli. A temperate coliphage, HK022, which has a function nun which antagonizes N action by using sites and proteins N normally uses for anti-termination has provided another approach to understanding N action. Progress has been made in reconstructing, in vitro, the complex lambda DNA replication system. Most of the previously identified components have been purified, and their interactions (particularly those of the host factors dnaJ and K with lambda proteins O and P) are being studies. The purification of these known components will allow the in vitro reconstruction of the DNA replication system and identification of factors which may not have been identified genetically. It will also allow a more detailed analysis of the mechanism of DNA replication priming.

Two independent ongoing projects in the Molecular Genetics Section (B. Howard) involve investigation of novel modes of mammalian growth regulation. Dr. Bruce Howard is using gene transfer to probe for human DNA sequences that control cell growth in an inhibitory mode. His results suggest that growth inhibitory sequences may be activated by an epigenetic mechanism in states of cellular quiescence and senescence. Dr. Gilbert Smith is continuing to characterize alterations in expression of mouse mammary tumor virus (MMTV), casein, and α-lactalbumin genes in the C3H/Sm mouse mammary gland tumorigenesis model. Recent experiments indicate that proteins coded by the MMTV long terminal repeat have DNA binding and dsDNA unwinding activities.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08000-15 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Gene Activity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Ira Pastan Chief, Laboratory of Molecular Biology NCI

COOPERATING UNITS *(if any)*

University of Oklahoma
Institute of Virology, Tokyo, Japan

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	4.8	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

cDNA clones coding for the epidermal growth factor receptor were isolated from a cDNA library prepared from A431 cells, a human epidermoid carcinoma. The total sequence of one of these clones which codes for the midportion of the EGF receptor has been determined. Using this clone, it has been shown that the EGF receptor is 30-fold amplified in A431 cells and also amplified in other squamous cell carcinomas. The promoter region of the EGF receptor has been identified and its DNA sequence determined.

PHS 6040 (Rev. 1/84) GPO 914-918

Other Professional Personnel:

G. Merlino	Staff Fellow	LMB, NCI
Y.-H. Xu	Visiting Fellow	LMB, NCI
S. Ishii	Visiting Fellow	LMB, NCI
A. Clarke	Guest Researcher	LMB, NCI
B. Roe	University of Oklahoma	
T. Yamamoto	Institute of Virology Tokyo University, Japan	

Project Description

Objectives:

To understand the molecular basis of cancer and growth control.

Methods Employed:

Prepare cloned cDNAs and isolate genes to use to study messenger RNA synthesis in intact cells or cell-free systems. Isolate animal cells that have mutations in the control of cell growth. Determine the biochemical basis of these alterations.

Significance for Cancer Research and the Program of the Institute:

If mutant genes can be identified in human cancers, it may be possible to modify these genes to control cancer.

Proposed Course:

Investigate the structure and function on the EGF receptor gene in various human tumors and to study its role in development.

Major Findings:

To help establish the role of the EGF receptor gene in malignancy, we have examined DNA from many different tumors. We have found the gene to be amplified in many human squamous cell carcinomas. These studies suggest that overexpression of the epidermal growth factor receptor gene may have an important role in this type of cancer. We have also examined the regulation of the EGF receptor gene in various tumor cell lines which do not contain amplified genes. We find there is a very good correlation between the amount of EGF receptor present in a cell and the amount of messenger RNA for that protein. Some cancer cells, such as melanoma cells, have no EGF receptor detectable and no detectable messenger RNA.

To understand how the EGF receptor expression is regulated, we have isolated the promoter region of the gene. The promoter region has several very interesting and unusual features. It does not contain a TATA box. Instead, it has five repeats of the sequence CCGCC. There are several sites of initiation of transcription

within the gene generating messenger RNAs of different lengths. The structure of the EGF receptor gene is very similar to that of the SV40 promoter and a few other promoters. There may be commmon structural features in essential genes that are subject to regulation related to growth control.

Two substances have been found which activate EGF receptor gene expression. One of these is EGF itself which initially down regulates the receptor. About one to two hours after EGF addition, messenger RNA levels begin to rise and remain elevated as long as EGF is present in the medium. A second substance which activates expression is PMA. This substance also induces EGF receptor internalization, but the receptor then reappears on the surface undegraded. Thus two substances which induce EGF receptor internalization stimulate receptor synthesis.

Publicatiions:

Xu, Y-h., Ishii, S., Clark, A., Sullivan M., Wilson, R.K., Ma, D. P., Roe, B., Merlino, G.T., and Pastan, I.: Human epidermal growth factor receptor cDNA in A431 carcinoma cells. Nature 309: 806-810, 1984.

Xu, Y.-h., Richert, N., Ito, S., Merlino, G., and Pastan, I.: Characterization of epidermal growth factor receptor gene expression in malignant and normal cell lines. Proc. Natl. Acad. Sci. USA 81: 7308-7312, 1984.

Merlino, G.T., Xu, Y.-h., Richert, N., Clark, A., Ishii, S., Banks-Schlegel, S., and Pastan, I.: Elevated epidermal growth factor receptor gene copy number and expression in a squamous carcinoma cell line. J. Clin. Invest. 75: 1077-1079, 1985.

Merlino, G.T., Ishii, S., Whang-Peng, J., Knutsen, T., Xu, Y.-h., Clark, A., Stratton, R., Wilson,, R., Ma, D.R., Roe, B.A., Hunts, J., Shimizu, N. and Pastan, I.: Structure and localization of genes encoding aberrant and normal epidermal growth factor receptor RNAs from A431 human carcinoma cells. Molecular and Cellular Biology. In press.

Merlino, G.T., Xu, Y.-h., Richert, N., Ishii, S., Clark, A., Stratton, R., Wilson, R.K., Ma, D.P., Roe, B.A., and Pastan, I.: Cloning and characterization of human epidermal growth factor receptor gene sequences in A431 carcinoma cells. In Cancer Cells. Cold Spring Harbor Press, Cold Spring Harbor, New York. In press.

Ishii, S., Xu, Y.-h., Stratton, R., Roe, B., Merlino, G.T., and Pastan, I.: Characterization and sequence of the promoter region of the human epidermal growth growth factor receptor gene. Proc. Natl. Acad. Sci. USA. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08001-14 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Cyclic AMP and Transforming Viruses in the Regulatin of Cell Behavior

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ira Pastan	Chief, Laboratory of Molecular Biology	NCI
OTHER:	M. M. Gottesman	Chief, Molecular Cell Genetics Section	LMB, NCI

COOPERATING UNITS *(if any)*

Department of Biochemistry, University of Massachusetts Medical School
Department of Cardiology, University of Chicago Medical School

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Insulin receptor is a substrate for src kinase. Cholera toxin treatment increases tumor formation by Rous Sarcoma virus transformed cells.

PHS 6040 (Rev. 1/84)

GPO 914-918

Other Professional Personnel:

D. Werth	Staff Fellow	LMB, NCI

Project Description

Objectives:

To understand the role of cyclic AMP and oncogenic viral gene products in malignant transformation and particularly how these factors regulate cell growth, cell morphology and cell adhesion.

Methods Employed:

Cell culture, viral transformation and standard biochemical analyses of the enzymes involved in cyclic AMP metabolism, protein phosphorylation, and the synthesis of macromolecules. Preparation of antibodies against transforming proteins. Purification of proteins.

Major Findings:

In collaboration with M. Czech, University of Massachusetts, we have shown that purified src kinase phosphorylates purified preparations of the insulin receptor and changes the activity of the receptor. This finding is in keeping with the possibility that insulin receptor activity in cells may be modulated by tyrosine phosphorylation.

With Michael Gottesman, it was shown that treatment of certain cells with cholera toxin enhances their ability to form tumors in nude mice. This finding raises the possibility that an enzyme of cyclic AMP metabolism may function as an oncogene.

Significance for Cancer Research and the Program of the Institute:

National Cancer Plan Objective 3, Approaches 1, 2 and 5: Objective 4, Approach 2, and Objective 6, Approach 3.

Various aspects of this work will lead to a better understanding of how cells become cancer cells and how the growth of cancer cells is controlled. It also has therapeutic implications.

Proposed Course:

To investigate new substrates for src kinase.

Publications:

Pastan I.: Citation Classic. Current Contents 27: 21, 1984.

Gottesman, M.M., Roth, C., Vlahakis, G., and Pastan, K.: Cholera toxin stimulates tumor formation by Rous sarcoma virus transformed cells. Mol. Cell Biol. 4: 2639-2642, 1984.

Yu, K-T., Werth, D.K.,.Pastan I., and Czech, M.: Src kinase catalyzes the phosphorylation and activation of the insulin receptor Kinase. J. Biol. Chem. 260: 5838-5846, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08006-14 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Control of Gene Expression in Bacteriophage Lambda

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, leboretory, and institute affiliation)*

PI:	M. Gottesman	Chief, Biochemical Genetics Section	LMB, NCI
OTHER:	S. Lu	Visiting Fellow	LMB, NCI
	G. Gaitanaris	Visiting Fellow	LMB, NCI
	N. Jahan	Visiting Fellow	LMB, NCI
	S. Sullivan	Biologist	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are continuing our study on the nature of transcription termination in E. coli and of the mechanism of action of the bacteriophage lambda N protein, that suppresses transcription termination.

We are constructing two new vector systems. The first of these is designed to clone DNA fragments bearing promoters active in E. coli. The second, a shuttle vector, is tailored for the cloning of large, and/or unstable DNA fragments, as well as for the reconstitution of genes from overlapping DNA clones.

We are examining the ability of coliphage P1 to stimulate the precise excision of transposons. The responsible gene has been subcloned, and we are attempting to clarify the molecular mechanism of this reaction.

We are studying gene regulation in the temperate coliphage HK022. HK022 antagonizes the action of the λN protein. We are also studying the control of bacteriophage lambda cII activity by phage and host functions.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

Our objective is to understand how DNA recombination and gene regulation is changed when E. coli carries an active λ or P phage. One goal of this work is to design vector systems, based on E. coli and phage λ, useful for the cloning and expression of a variety of prokaryotic and eukaryotic genes.

Methods Employed:

Standard microbial genetic and biochemical techniques required for plasmid phage and bacterial constructions, and for enzyme assays.

Major Findings:

We have cloned and sequenced a phage P1 gene (ref) that stimulates the precise excision of a transposon. The sequence is unusual in that no ribosome binding site precedes the gene. We have introduced a ribosome binding site and have synthesized large amounts of Ref (about 50% of the cell protein is Ref) using an expression vector.

We have studied the stability and synthesis of the lambda cII protein. The synthesis of this highly unstable protein requires the E. coli histone-like protein, IHF. We have shown that the IHF is needed to translate the cII gene and have determined the relevant cII sequences involved in this regulation. We are collaborating with Dr. Amos Oppenheim in this study.

The temperate coliphage, HK022, blocks the growth of phage lambda by inducing premature transcription termination on the lambda genome; it thus antagonizes the lambda N protein. We have determined (in collaboration with Dr. Robert Weisberg) that a HK022 function, nun, terminates transcription at the lambda nut sequences and uses the E. coli Nus proteins to promote this reaction. We have subcloned nun and isolated lambda mutants resistant to the effects of nun.

Mutations in the E. coli gene "u" suppress the E. coli nusA1 mutation for lambda growth. We have isolated plasmid and vector "u" clones and selected a "u" amber mutation.

Significance to Biomedical Research and the Program of the Institute:

National Cancer Plan Objective 3, Approach 1.

In cancer cells, the expression of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using λ as a model system. This understanding might help to prevent the conversion of normal cells to those capable of forming cancers.

Proposed Course of Research:

We are using the promoter cloning vector to study the regulation of promoters and to obtain mutations that alter this regulation. The system offers, as well, the possibility of obtaining promoters that initiate nonterminating transcripts and to investigate the factors that regulate such promoters.

We will isolate the ref gene product and attempt to study its *in vitro* properties.

We will further analyze the mechanism of transcription termination by isolating phage and host mutations that influence this reaction.

We will clone and sequence the HK022 *nun* gene, and attempt to isolate its product. We will characterize the lambda *nun* resistant mutants. We will isolate additional host mutations which block the action of *nun*.

We will continue our investigation of the regulation of cII expression by IHF. We will probe the cII regulatory region with *in vitro* mutagenesis. Our model of IHF action makes direct predictions about the structure of cII mRNA; we will test these predictions *in vitro* using RNAase probes.

Publications:

Rattray, A., Altuvia, S., Mahajna, G., Oppenheim, A.B., and Gottesman, M.: Control of bacteriophage lambda cII activity by bacteriophage and host functions. *J. Bacteriol.* 159: 238-242, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08010-12 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Morphologic Mechanisms of Organelle Function and Transformation in Cultured Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M. C. Willingham, Chief, Ultrastructural Cytochemistry Section LMB, NCI

COOPERATING UNITS *(if any)*

Department of Pharmacology and Experimental Therapeutics
Johns Hopkins University School of Medicine, Baltimore, Maryland

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Ultrastructural Cytochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Neoplastic transformation produces many changes in cell physiology. Growth-promoting hormones produce some of these same changes. Endocytosis is a process that regulates both the interaction of cells with growth-promoting hormones and the entry of transforming viruses. In the last few years, our study of the pathway of endocytosis in cultured cells has revealed that epidermal growth factor (a growth promoting hormone)(EGF) and transferrin (a plasma iron-binding protein necessary for cell growth)(TF) are co-internalized in the same pathway into human carcinoma cells, but diverge from each other in the trans-reticular network of the Golgi system. Cytochemical experiments using electron microscopy have shown that the receptors for EGF and TF are also internalized with the ligands. However, EGF and its receptor have been found to be delivered to lysosomes and degraded, whereas TF and its receptor are recycled intact back to the cell surface. The morphologic divergence of these two ligand-receptor types appears to involve clathrin-coated pits of the Golgi system. We have now shown that cell fractionation using an anti-clathrin affinity absorbant technique revealed the same separation of TF and EGF seen morphologically. It was also shown that the entry of the EGF receptor could be at least partially induced by treatment of cells with phorbol esters in the absence of EGF. To evaluate the site of ligand-receptor sorting, we employed new markers for the Golgi and lysosomal systems, including: (1) Limax flavus lectin labeling of sialic acid residues in the trans-Golgi system, (2) LAMP-1 monoclonal antibody to a lysosomal membrane protein, (3) ABL-70 monoclonal antibody to a Golgi stack marker protein, (4) antibody to MEP as a marker of lysosomal matrix proteins, and (5) 2C6 monoclonal antibody as a marker for the alpha$_2$-macroglobulin receptor. We examined the morphologic kinetics of delivery of ligand into the lysosomal system in double-label experiments using LAMP-1 as a marker for lysosomal membranes. We also devised a new method for preservation of membrane proteins for immunofluorescence detection.

Other Professional Personnel:

I. H. Pastan	Chief, Laboratory of Molecular Biology	LMB, NCI
M. M. Gottesman	Chief, MCG Section	LMB, NCI
J. Hanover	Guest Worker	LMB, NCI
L. Beguinot	Guest Worker	LMB, NCI
R. Lyall	Guest Worker	LMB, NCI
K. Goldenthal	Medical Staff Fellow	LMB, NCI
K. Hedman	Fogarty Fellow	LMB, NCI
J. T. August	Professor, Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine	

Project Description

Objectives:

To investigate the mechanisms that control basic cell organelle functions such as endocytosis, exocytosis, intracellular protein traffic, viral infection, and the morphologic and growth manifestations of malignant transformation.

Methods Employed:

Cell culture, specialized light and electron microscopic morphologic and cytochemical methods including ultrastructural immunocytochemical localization procedures, hybridoma production of monoclonal antibodies, and specialized biochemical and analytical methods.

Major Findings:

The endocytosis of receptor-bound ligands such as growth-promoting hormones and nutrient factors follows a common pathway in cultured mammalian cells. The initial step for many of these ligands after binding to their receptor is the concentrative clustering of receptor-ligand complexes in clathrin-coated pits of the cell surface. This is followed by endocytosis into a special endocytic vesicle we have previously described, termed a receptosome. These receptosomes migrate to the trans-reticular elements of the Golgi system with which they fuse. Many ligands are delivered to the TR Golgi; some are directed to lysosomes, whereas others are directed to exocytosis for recycling back to the cell surface. We have examined the segragation of these pathways of delivery of both ligands and their receptors using epidermal growth factor (EGF) and its receptor as markers for the delivery to lysosomes, and transferrin (TF) and its receptor as markers of the delivery back to the cell surface. We have used conjugates of the ligands and immunocytochemical localization of the receptors to examine the morphologic location of these sorting events. The separation of these two marker ligands appears to occur in the trans-reticular (TR) Golgi system. Morphologically, the coated pits of the TR-Golgi appear to concentrate EGF prior to its delivery to lysosomes, but to exclude transferrin. This morphologic observation has been confirmed using an affinity absorbant of

anticlathrin antibody-Sepharose and cell fractionation. In addition, studies in which the EGF receptor was localized using monoclonal and polyclonal antibodies to different domains of the EGF receptor have shown that phorbol esters can induce a portion of the EGF receptor population to be internalized in the absence of EGF, suggesting a possible role for phosphorylation in the induction of receptor clustering and entry.

We have also utilized monoclonal antibodies and other cytochemical labels to identify specific organelle domains in cells that are involved in this endocytic-exocytic pathway. Limax flavus (slug) lectin was used to detect the parts of the Golgi system that contain sialic acid glycoconjugates as a marker of the trans-elements of the Golgi. This study showed that the TR Golgi elements involved in sorting and exocytosis both contained large amounts of sialic acid, implicating the TR elements involved in endocytic sorting as being part of the Golgi system. LAMP-1 is a monoclonal antibody that detects a 110K glycoprotein that we have localized exclusively to the lumenal face of lysosomal membranes. It serves as a specific marker for the entry of ligands into the lysosomal system, and in double-label experiments using alpha$_2$-macroglobulin-colloidal gold, the kinetics of entry of the ligand into these lysosomal elements was precisely mapped. ABL-70 is a monoclonal antibody that we have shown reacts selectively with an element of the Golgi stack system, serving as a selective marker to distinguish stack from trans-reticular Golgi elements; none of the ligands internalized by endocytosis appear to co-localize with this Golgi stack marker. MEP has been localized to lysosomes as a matrix protein tentatively identified as a lysosomal enzyme, and serves as a marker for lysosomal content. In transformation this protein has been shown to be produced in such large quantities that much of it is secreted by exocytosis, also suggesting a common origin for intracellular lysosomal and exocytic pathways from trans-Golgi elements. 2C6 is a monoclonal antibody that detects a protein tentatively identified as the receptor for alpha$_2$-macroglobulin which we have localized in receptosomes and Golgi elements in the pathway of receptor recycling, but not in lysosomes where the ligand for this system (alpha$_2$-M) is delivered. We have used some of these markers to examine the possible common pathway of a recycling ligand (transferrin) and an exocytic membrane protein (VSV "G" protein) derived from Golgi stacks using the O-45 mutant of VSV. In these double-label studies, the organelles containing "G" protein and recycling transferrin appear to follow similar morphologic pathways to the cell surface, although direct fusion of the vesicular elements in these two pathways has not been clearly demonstrated. Studies on the fixation of these various membrane proteins using monoclonal antibodies has shown that many of these proteins are poorly fixed by formaldehyde fixatives, and their detection by immunofluorescence is dramatically impaired by detergents such as Triton X-100 after formaldehyde fixation; the substitution of saponin for Triton X-100 produces significantly better preservation and provides a generally useful alternative method for the immunofluorescence detection of membrane proteins.

Significance to Cancer Research and the Program of the Institute:
National Cancer Plan Objective 6, Approach 3.

Transformation of cultured cells appears closely linked with the ability to form malignant tumors in vivo. The understanding of the basic mechanisms that

regulate cell movement, organelle function, response to growth-promoting hormones, virus infection, and the morphologic changes that occur following transformation are likely to be of great value in understanding the basic mechanisms that are altered in most cancer cells. In particular, the understanding of the mechanisms of endocytosis is potentially of great importance in the proper design and use of immunotherapeutic reagents, such as immunotoxins. This knowledge is likely to have significant impact on the ability to design successful therapeutic procedures for malignancy.

Proposed Course:

We will continue to study the basic cellular mechanisms that regulate the movement and function of organelles and cells, the entry and exit of cellular proteins, viruses, and hormones, and the interactions of specific transformation-linked molecules with these systems. Through a study of the alterations in cell function related to transformation, we will try to gain a more precise understanding of the mechanisms by which cancer cells are able to grow uncontrollably and metastasize.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08011-11 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structures and Roles of Transformation-Sensitive Cell Surface Glycoproteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K. M. Yamada	Chief, Membrane Biochemistry Section	LMB, NCI
OTHER:	S. K. Akiyama	Guest Researcher	LMB, NCI
	M. J. Humphries	Guest Researcher	LMB, NCI
	K. Olden	Guest Researcher	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Membrane Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.7	2.7	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The glycoprotein fibronectin is often decreased on tumor cells and is involved in cell adhesion and migration. Its two major forms, termed plasma and cellular fibronectins, were shown to be very closely related structurally within a species, but much less between species, by immunological and peptide mapping criteria. This pattern indicates an origin from a single gene rather than a gene family. The site on these fibronectins that interacts with cells was explored using synthetic peptides from evolutionarily conserved regions. The sequence Gly-Arg-Gly-Asp-Ser was found to be a critical recognition signal in fibroblast adhesion assays. Variant peptides generally showed much less activity, with the sole exception of the short inverted tetrapeptide sequence Ser-Asp-Gly-Arg. Fibronectin peptides were also found to be functional *in vivo*, and microinjection into amphibian or chicken embryos specifically blocked gastrulation and neural crest cell migration. These and other preliminary experiments suggest that these peptides may also inhibit tumor cell migration and invasion. Methods for directly quantitating fibronectin binding by cells were developed using tritiated fibronectin and cell-binding fragments. Fibronectin bound to cells with moderate affinity (K_d = 8 x 10^{-7}M) with up to 500,000 sites per cell. Synthetic peptides also competitively inhibited fibronectin binding to the cell surface, but with lower apparent affinity (K_i = 10^{-3} to 10^{-4}M). These results strongly suggest that other sequence information besides the critical pentapeptide sequence are needed for full binding activity. Our future objectives will be to define the elements of fibronectin primary structure besides the critical pentapeptide sequence that are involved in binding to the cell surface, to explore the generality of this adhesive recognition signal in a variety of cell-to-substrate and cell-to-cell adhesion systems, especially in malignant cells, and to establish whether *in vivo* treatment with fibronectin synthetic peptides can prevent tumor cell invasion and metastasis in model systems.

Project Description

Objectives:

Fibronectin is a major cell surface and extracellular matrix glycoprotein that is often decreased after malignant transformation, and is involved in cell adhesion and migration.

Our objectives are:

(1) To elucidate the functions of fibronectin and other adhesion proteins and to determine their mechanisms of action.

(2) To characterize the molecular features of fibronectin and other extracellular molecules that govern their interactions with cells.

(3) To develop specific, non-toxic inhibitors of the function of fibronectin and other adhesive proteins to disrupt cell adhesion, migration, invasion, and/or metastasis.

Methods Employed:

Cellular fibronectins from human, mouse, and chicken fibroblasts were isolated by urea extraction, ammonium sulfate fractionation, and gelatin affinity chromatography. Plasma fibronectins were purified from citrated plasmas by gelatin-Sepharose affinity chromatography and elution either under non-denaturing conditions by 50 mM sodium citrate, pH 5.5, or by elution and further purification in urea using heparin-Sepharose columns. The cell-binding fragment of fibronectin was generated by controlled tryptic degradation of human fibronectin, and was purified by sequential DEAE, gelatin-affinity, and heparin-affinity chromatography steps; its purity was confirmed by SDS gel electrophoresis. Synthetic peptides were synthesized according to our specifications by Peninsula Laboratories and Vega, then further purified by Fractogel TSK HW40S chromatography and reversed phase HPLC on a preparative C18 bonded silica column. Purity was assessed by reversed phase HPLC and quantitative amino acid analysis.

Structural domains of fibronectins were compared after partial tryptic digestion of native molecules with TPCK-trypsin at 2 µg/ml. Peptide mapping was performed after iodination with chloramine T and exhaustive digestion of SDS-denatured, electrophoretically purified fibronectins with TPCK-trypsin. Peptides were separated by electrophoresis on thin layer cellulose plates, follwed by chromatography in butanol/acetic acid/pyridine/water. Immunological comparisons were performed with the ELISA procedure using wells coated with 2 µg/ml of each type of purified fibronectin, followed by goat anti-fibronectin antisera directed against human or chicken fibronectins, then rabbit anti-goat IgG coupled to horseradish peroxidase.

For cell adhesion assays on fibronectin, a 3 µg/ml solution of plasma fibronectin was incubated with tissue culture dishes for one hour. Non-specific adsorption sites were blocked by 30 min. incubation with 10 mg/ml heat-denatured bovine serim albumin (80°C for 3 min.). Competitive inhibition of cell adhesion

and spreading on plastic or type I collagen substrates was evaluated with 0.5-10 mg/ml purified, soluble fibronectin or with purified synthetic peptides at 5-1000 µg/ml.

For receptor binding studies, purified human plasma fibronectin or its 75,000 dalton cell-binding fragment were labeled by reductive methylation using tritiated sodium borohydride and formaldehyde. Labeled ligands were incubated with or without competing unlabeled ligands with BHK cells in serum-free Dulbecco's medium at high cell density ($0.5\text{-}4 \times 10^7$ cells/ml) at 4°, 22°, and 37°C. Bound radioactivity was determined after washing and centrifugation through a layer of 10% bovine serum albumin. Non-specific binding was determined in the presence of an 80-to-100 fold excess of unlabeled ligand.

Major Findings:

Fibronectin is a major cell surface and extracellular glycoprotein which is considered to be a prototype cell adhesion molecule involved in cell migration. We have been exploring the structural basis of its activities, focusing on the elements essential for its interaction with cells and for normal and malignant cell adhesion, migration, and invasion. The two major forms of fibronectin, cellular and plasma fibronectins, obtained from three different species were compared for structural similarities and differences. Limited or exhaustive tryptic digestion revealed highly homologous protease-resistant domains and peptides from the two forms within species, but few homologies between species, even though the functional activities were similar. This pattern of similarities and differences was confirmed by quantitative immunological comparisons. These results support a model of derivation of these two major fibronectin isoforms from the same gene, whose sequence drifts during evolution.

The information on fibronectin needed for interactions with cells was examined in a newly established assay system for direct binding to the cell surface. Binding of fibronectin was found to occur with moderate affinity, with a K_d of 8×10^{-7} M and as many as 5×10^5 binding sites per cell. Binding did not appear to require interaction of the fibronectin with a substrate, apparently ruling out current models postulating a requirement for a substrate activation step.

The amino acid sequence specificities of the site on fibronectin critical for attachment to the cell surface were defined further using synthetic peptides that specifically and competitively inhibited cell adhesion. The most active sequence was found to be the pentapeptide Gly-Arg-Gly-Asp-Ser. The tetrapeptide Arg-Gly-Asp-Ser was found to retain substantial activity, but was three-fold less active. An "inverted" peptide sequence in which these same four amino acids were arranged in the mirror-symmetrical order Ser-Asp-Gly-Arg was found to be nearly as active as the forward sequence. However, if this same sequence was embedded in a synthetic decapaptide derived from a conserved sequence from histocompatibility antigens, it had much less activity, suggesting the importance of adjacent sequence in modulating the activity of such peptides. Other permutations of the order or side chain composition of amino acids demonstrated the specificity of the basic pentapeptide sequence. A hypothesis was proposed that this adhesive recognition signal consists of a specific spatial arrangement of one acidic and one basic charged group, with additional information provided by adjacent amino acids. These fibronectin peptides were tested in the direct binding assay for

quantitating the binding of fibronectin to cells. They competitively inhibited binding, as demonstrated by double reciprocal plots, indicating a direct interaction at the same receptor site. However, the inhibition was of lower affinity than intact fibronectin ($K_i = 10^{-3}$ to 10^{-4} M), indicating that additional information promoting efficient binding is present elsewhere in the fibronectin molecule.

Synthetic fibronectin peptides were then tested as inhibitors of morphogenetic cell migration. The peptides specifically inhibited the migration of neural crest cells in vitro, whereas, another peptide from a conserved sequence in the collagen-binding domain of fibronectin had no effect. Microinjection of the peptides in vivo resulted in specific inhibition of chick neural crest cell migration, as well as inhibition of amphibian gastrulation. These studies establish such peptides as useful inhibitors and probes of migratory events in vivo. Studies presently in progress are exploring the use of these peptides as inhibitors of tumor cell invasion and metastasis. Preliminary experiments indicate that large single doses are non-toxic in mice, and that it may be feasible to inhibit metastasis in a standard murine melanoma model system.

Significance to Cancer Research and the Program of the Institute:

Fibronectin and other cell surface and extracellular molecules mediate the interactions of cells with connective tissue. Invasion and metastasis are thought to require adhesive and migration-related interactions with extracellular molecules. An adhesive recognition signal necessary for cell migration that may be shared by several adhesive molecules has been characterized. Synthetic peptides corresponding to this short amino acid sequence can be produced in large quantities, and they can serve as diffusable, competitive inhibitors that prevent these adhesive interactions in vivo. Such peptides, or other sequences crucial to cell adhesion and migration, may prove useful therapeutically as inhibitors of tumor cell invasion and metastasis.

Proposed Course of Research:

We propose to extend these studies focusing on adhesive recognition signals:

(1) To determine whether fibronectin synthetic peptides can efficiently inhibit tumor cell invasion and metastasis in model systems -- initially the standard B16 melanoma metastasis system -- by evaluating routes of administrations, the most effective dosages, and specificity. These experiments will be extended to include evaluation of clearance rates of peptide, mechanism of action, and general applicability to other in vitro invasion and in vivo metastasis sytems.

(2) To determine which systems of cell-to-substrate and cell-to-cell adhesion are dependent on the fibronectin-type adhesive recognition signal, and to determine whether malignant cells adhere to extracellular molecules by mechanisms that are similar to or distinct from those of normal cells.

(3) To continue quantitative comparisons of the activity of various fibronectin fragments to determine what information besides the minimal pentapeptide sequence is involved in fibronectin binding to cells and determines its specificity.

(4) To continue DNA sequencing of recombinant clones spanning the fibronectin gene to examine the 5' end of the mRNA and the regulatory region of the gene.

(5) If time permits, to begin evaluation of functionally important sites in the collagen-binding region of fibronectin using chemical cross-linking to the purified CB7 fragment of collagen.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08700-13 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Expression of Collagen Genes in Normal and Transformed Cells

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: B. de Crombrugghe, M.D. Chief, Gene Regulation Section, LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.0	5.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the spece provided.)*

(1) A functional analysis of deletions in the promoter of the mouse α_2(I) collagen gene indicates there are two different segments far upstream of the start of transcription that are important for optimal expression of this gene.

(2) Transgenic mice were generated in which an α_2(I) collagen promoter-chloramphenicol acetylase chimeric gene has been stably introduced in the germline. These new mouse strains show a tissue specific pattern of expression for the chimeric gene that coincides with that of the endogenous type I collagen genes.

(3) Mutant NIH 3T3 fibroblasts have been obtained in which type I collagen and fibronectin genes escape from the transcriptional control which normally inhibits their expression in v-mos transformed cells.

(4) A segment with dyad symmetry around the translation initiation site of α_2(I) collagen mRNA exerts a strong inhibition on the level of translation of this mRNA.

(5) The presence of v-fos in NIH 3T3 cells strongly stimulates expression of the α_1(III) collagen gene. This stimulation is mediated by transcriptional activation of the promoter for this gene.

PHS 6040 (Rev. 1/84) GPO 914-918

Other Professional Personnel:

M. Mudryj	Chemist	LMB, NCI
A. Schmidt	Visiting Associate	LMB, NCI
G. Liau	Guest Researcher	LMB, NCI
C. Setoyama	Visiting Fellow	LMB, NCI
P. Rossi	Guest Researcher	LMB, NCI
D. Szapary	Guest Researcher	LMB, NCI
A. Hatamochi	Visiting Fellow	LMB, NCI

Collaborators:

H. Westphal	Chief, Laboratory of Molecular Genetics	NICHD
J. Khillan	Visiting Fellow	LMG, NICHD
P. Overbeek	Staff Fellow	LMG, NICHD
B. Peterkofsky	Research Chemist	LB, NCI

Project Description

Objectives:

(1) To understand the molecular mechanisms controlling the differentiation programs which determine the levels of expression of specific genes in animal cells.

(2) To understand the mechanisms by which the products of various oncogenes alter these differentiation programs.

(3) As a model system, we use the collagen genes, a series of developmentally regulated genes whose protein products form an important component of the extracellular matrix in most multicellular organisms. One of the experimental systems which we use to study the regulations of the type I and III collagen genes consists of cultured fibroblasts, both normal fibroblasts and fibroblasts transformed by tumor viruses or by oncogenes. After transformation by a series of oncogenes, several changes occur in these cells in the pattern of proteins synthesized. The synthesis of type I and type III collagen is greatly reduced in many transformed cells, and this decrease is mediated by a transcriptional control mechanism. One of our objectives is to understand the mechanisms which determine the tissue-specific and developmental expression of these genes as well as those which inhibit the expression of the type I and III collagen genes in many malignant transformed cells. More specifically, we want to identify cis-acting elements in the type I and III collagen genes and transacting cellular factors which determine the level of expression of these genes.

Methods Employed:

(1) Cloning of animal and bacterial genes in the DNA of bacterial plasmids and bacteriophage; construction of recombinant DNAs; construction and screening of cDNA and gene libraries.

(2) DNA transfection of animal cells, selection of transfected cells.

(3) Purification of specific DNA fragments and RNA species, nucleotide sequence analysis of DNA and RNA.

(4) Measurement of RNA and protein synthesis in vivo and in cell-free systems.

(5) Purification of factors active in regulation of gene expression.

(6) Mutagenesis of cells and site-specific mutagenesis of specific gene segments.

(7) Microinjections in tissue culture cells and mouse oocytes, generation of transgenic mice.

Major Findings:

(1) Functional analysis of the cloned α_2(I) promoter. We have constructed a number of recombinant plasmids to study the activity of this promoter after DNA mediated transfection of mouse fibroblasts. In these plasmids, the α_2(I) collagen promoter has been placed at the 5' end of a marker gene. As marker genes we have used either the baterial gene for chloramphenicol acetyl transferase or the bacterial gene which confers resistance to an analog of neomycin called G418. The constructions also contain splice sites and the poly(A) addition site of the early region of SV40, the bacterial β-lactamase gene and the origin of replication of ColE1.

A deletion analysis of this promoter indicates that at least two separate segments are important for optimal expression of this promoter. One is located between -900 and -498, the other between -342 and -98. A more detailed analysis of the second segment shows that this segment may interact with both an activator and a repressor.

We have used a similar chimeric gene which consists of the CAT gene fused to a segment of the α_2(I) collagen promoter (from 2,000 bp upstream of the start of transcription to 54 bp downstream of this site) to show that the collagen promoter segment is sufficient for cell specific expression, since its expression in fibroblasts, cells that make type I collagen, is much higher than in cell types which do not synthesize type I collagen.

Finally, in NIH 3T3 cell lines, in which the chimeric gene consisting of the α_2(I) collagen promoter-CAT unit was stably integrated, subsequent transformation by v-mos strongly inhibits the expression of the chimeric gene in parallel to the inhibiton of the endogenous type I collagen genes.

(2) Development of a genetic system to study the regulation of type I collagen genes. We have observed that a construction containing the α_2(I) collagen promoter fused to the neomycin resistance gene introduced into mouse NIH 3T3 cells confers resistance to G418. After transformation by v-mos, many of these cells become sensitive to G418. We have mutagenized two such G418 sensitive cell lines to select for G418 resistant cells. Our experiments indicate: (a) that mutants are obtained but only after mutagenesis;

(b) that their frequency is in the order of 10^{-5} to 10^{-6}; (c) that several classes of mutants were obtained; (d) the most interesting mutants are two pleiotropic mutants which show not only increased levels of neo RNA, but also increased levels of type I collagen RNA and increased amounts of secreted fibronectin, whereas, the levels of mos RNA are unchanged. Since the mutant cells are able to induce tumors in nude mice with the same kinetics as the parent cells and since their levels of v-mos RNA are unchanged, it is likely that they contain the same levels of functional v-mos. We proposed that the pleiotropic phenotype of these mutant cells could be due to a mutation in a transacting cellular factor which controls, either directly or indirectly, expression of the type I collagen and fibronectin genes. These mutants are able to escape from the control which normally inhibits the expression of these genes in v-mos transformed cells.

(3) Transgenic Mice. Eight transgenic mice were generated, in which the promoter of the mouse α_2(I) collagen gene (-2000 to +54) linked to the bacterial gene for chloramphenicol transacetylase, is stably integrated in the germline. These strains contain one or more copies of the α_2(I) collagen-CAT DNA unit integrated presumably at a single site in the mouse genome. Although the copy number of the α_2(I) collagen promoter CAT unit and its site of integration in the genome vary from one strain to the other, these properties are stably transmitted to the progeny of the different strains following a classical Mendelian segregation pattern. In seven of the eight strains, the CAT gene is expressed, although the levels of CAT enzyme activity vary considerably from one strain to the other. In six of these strains, the expression of the CAT gene follows the expected tissue distribution pattern of expression of the α_2(I) collagen gene. In these six strains the level of CAT activity is much higher in extracts of tail, a tissue that is very rich in tendons, than in any other tissue that was tested. This distribution parallels the much higher levels of α_2(I) collagen RNA that are found in the tail as compared to other tissues. We conclude that the α_2(I) collagen promoter sequences present in the recombinant plasmid used for our experiments contain the necessary information to ensure tissue-specific activity of this promoter.

(4) Translational control of the type I and III collagen genes. A chimeric gene was constructed in which a segment containing the α_2(I) collagen promoter (from -2000), the 5' untranslated region, and the sequences coding for the promoter-proximal part of the signal peptide, is linked to the chloramphenicol acetyltransferase gene. In this construction, the beginning of the signal peptide is fused to the CAT polypeptide in an in-frame protein fusion. Two different deletions which remove small sequences that include part of a potential stem-loop structure cause a 20-fold increase in CAT expression after DNA transfection of NIH 3T3 cells. Using these recombinant plasmids, we have obtained evidence that is consistent with a feedback control mechanism for translation control that could involve the amino-propeptide extensions of type I collagen.

(5) V-fos induces type III collagen in fibroblasts. In contrast to transformation by mos, Ras or src which results in a strong inhibition of type I collagen synthesis, transformation by v-fos does not change the levels of type I collagen but strongly stimulates synthesis of type III collagen. This

stimulation is mediated by a transcriptional control. Since levels of v-fos increase in other cell types during differentiation, we propose that v-fos may redirect the differentiation program of NIH 3T3 cells.

Proposed Course:

(1) Pursue the functional analysis of the α_2(I) collagen promoter and perform similar experiments with the α_1(III) collagen promoter. Determine by oncogenes. Search for additional enhancing sequences in the α_2(I) and α_1(III) collagen genes.

(2) Isolate the mutated gene(s) in our NIH 3T3 mutants which escape from the inhibition of type I collagen and fibronectin synthesis imposed by v-mos. Isolate additional mutants.

(3) Obtain transgenic mice in which segments of the α_2(I) collagen promoter have been deleted to determine which sequences are responsible for tissue-specific and developmental regulation of the gene. Establish new transgenic mice containing a full-length cDNA for one of the collagen genes. Introduce deletions at specific locations in this cDNA and microinject the deletions into mouse oocytes. Use these transgenic mice as a model system to study genetic diseases affecting collagen genes.

(4) Isolate, purify and characterize factors that bind to the promoter region of the type I and III collagen gene. Examine differences between extracts from different type of cells, including normal and transformed fibroblasts.

(5) Establish under which physiological conditions translational control of the type I and III collagens is observed. Initiate studies to understand the biochemical mechanisms of this translational control.

Publications:

Schmidt, A., Yamada, de Crombrugghe, B.: DNA sequence comparison of the regulatory signals at the 5' end of the mouse and chick α_2 type I collagen genes. J. Biol. Chem. 259: 7411-7415, 1984.

McKeon, C., Schmidt, A., de Crombrugghe, B.: A sequence conserved in both the chicken and mouse α_2(I) contains sites sensitive to S_1 nuclease. J. Biol. Chem. 259: 6636-6640, 1984.

de Crombrugghe, B., Busby, S., and Buc, H.: Activation of transcription by the cyclic AMP receptor protein. In Goldberger, R.F., and Yamamoto, K.R. (Eds.): Biological Regulation and Development. New York, Plenum Press, 1984, pp. 129-167.

Yamada, Y., Liau, G., Mudryj, M., Obici, S., de Crombrugghe, B.: Conservation of the sizes for one but not another class of exons in two chick collagen genes. Nature 310: 333-337, 1984.

Liau, G., Yamada, Y., and de Crombrugghe, B.: Coordinate regulation of the levels of type III and type I collagen mRNA in most but not all mouse fibroblasts. J. Biol. Chem. 260: 531-536, 1985.

Schmidt, A., Setoyama, C., and de Crombrugghe, B.: Regulation of a collagen gene promoter by the product of viral mos oncogene. Nature 314: 286-289, 1985.

Liau, G., Mudryj, M., and de Crombrugghe, B.: Identification of the promoter and first exon of the mouse α_1(III) collagen gene. J. Biol. Chem. 260: 3773-3777, 1985.

de Crombrugghe, B., Schmidt, A., Liau, G., Setoyama, C., Mudryj, M., Yamada, Y., and McKeon, C.: Structural and functional analysis of the genes for α_1(I) and α_1(III) collagens. Annals of the New York Academy of Sciences. In press.

de Crombrugghe, G., Liau, G., Setoyama, C., Schmidt, A., McKeon, C., and Mudryj, M.: Structural and functional studies on the interstitital collagen genes. Ciba Symposium on Fibrosis. In press.

Setoyama, C., Liau, G., and de Crombrugghe, B.: Pleiotropic Mutants of NIH 3T3 cells with altered regulation in the expression of both type I collagen and fibronectin. Cell. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08704-32 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Thyroid Growth and Involution

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

PI:	S. H. Wollman	Chief, Cell Organization Section	LMB, NCI
OTHER:	C. Tacchetti	Visiting Fellow	LMB, NCI
	L. Nitsch	Istituto di Patologia Generale University di Napoli, Naples, Italy	
	C. Garbi	Istituto di Patologia Generale University di Napoli, Naples, Italy	

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Cell Organization Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have found that thyroid epithelial cells in inverted follicles transport H_2O at rates of about one-sixth the rate of kidney cells.

We have found that when the apical surface of thyroid epithelial cells begin to adhere to the surface of a collagen gel, it is by a specialized region of the surface, the site of a pseudopod and not of microvilli.

We have identified cells migrating as single cells from follicles embedded in or on a collagen gel to be fibroblasts that are trace contaminants of the original culture.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:
To study thyroid growth and differentiation and the properties of thyroid cells in separated follicles in suspension culture, including the influence of extracellular matrix on these properties.

Methods Employed:

Thyroid gland was dissociated by collagenase treatment. Small clusters of epithelial cells were isolated from the dissociated tissue by centrifugation followed by filtration through fine nylon mesh. Clusters were cultured in suspension. They round up around a lumen within one day. Properties were examined by phase microscopy, autoradiography and electron microscopy. Such follicles or inverted follicles were sometimes cultured on or within a collagen gel.

Major Findings:

Identification of cells that migrate from thyroid follicles embedded in a collagen gel (with C. Tacchetti).
We have tried to identify with greater certainty the cells that migrate as single cells from follicles embedded in a collagen gel. They are probably fibroblasts that were a trace contaminant in the culture. This was important to establish because on superficial examination the phenomenon appeared to be an example of an epithelial to mesenchymal cell conversion.

Water transport by thyroid epithelial cells (with L. Nitsch and C. Tacchetti).
We have estimated the rate of water transport through thyroid epithelial cells in follicles in suspension culture to be approximately 0.5 $\mu m^3/\mu m^2/min$.
This is about one-sixth the rate in MDCK cells in a monolayer.

Nature of primary adhension site to collagen of thyroid epithelial cells in inverted follicles (with C. Tacchetti).
Further study of the mechanism by which inverted follicles in suspension culture adhere to the surface of a collagen gel reveals that the primary adhesion site on the microvilli-bearing surface of the cell is a pseudopod extending into the medium from the cell surface. The different adhesion properties of the pseudopod and microvilli may be a reflection of their different surface charge since microvilli are labeled by cationized ferritin whereas the pseudopod is not.

Significance to Cancer Research and the Program of the Institute:
These studies are part of a program to determine the effects of extracellular matrix on normal thyroid cells.

Proposed Course:

Termination

Publications:

Wollman, S.H., Nitsch, L., Garbi, C., and Tacchetti, C.: Aspects of control of polarity of thyroid epithelial cells in follicles in suspension culture. In Eggo, M.C. and Burrow, G.N. (Eds.): Thyroglobulin--The Prothyroid Hormone. New York, Raven Press, 1985, pp. 235-242.

Tachiwaki, O. and Wollman, S.H.: Honeycomb structure of the apical surface of thyroid epithelium during involution of the hyperplastic thyroid gland. Exp. Cell Res. 158: 104-110, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08705-09 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic and Biochemical Analysis of Cell Behavior

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M. M. Gottesman Chief, Molecular Cell Genetics Section LMB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are utilizing the Chinese hamster ovary (CHO) fibroblast to study the genetics and biochemistry of some aspects of the behavior of cultured cells. Our work has emphasized morphology and its relationship to growth control, and response to cyclic AMP. We have isolated a variety of different mutants with altered microtubules which express mutated α- or β-tubulin subunits and used these mutants to study spindle formation and the mechanism of anti-microtubule drug action. These mutants are defective in spindle formation because the mutant tubulins are present in spindles and interfere with their normal function. One β-tubulin mutation has been transferred by DNA mediated gene transfer to wild-type cells where it is amplified, overexpressed and renders the cells dependent on Colcemid for growth at 37°. We have also established two cell systems for examining the ways in which AMP can positively and negatively regulate cell growth. CHO cell growth is inhibited by cAMP; mutants selected for resistance to growth inhibition have defective cAMP dependent protein kinases. We have used DNA from cells carrying dominant cAMP-resistant defects to tranfer the cAMP-resistance phenotype to sensitive cells and are cloning these genes from cosmid libraries prepared from our mutant cell lines. CHO cells malignantly transformed by RSV are also cAMP-resistant. Formation of tumors by CHO-RSV cells and human melanoma cells is dependent on prior treatment with cholera toxin which raises cAMP levels within the cells. This increased tumorigenicity is an example of positive regulation of cell growth by cAMP.

GPO 914-918

Other Professional Personnel:

G. Vlahakis	Research Biologist	LMB NCI
M. Chapman	Research Biologist	LMB NCI
R. Fleischmann	Staff Fellow	LMB NCI
R. Kuriyama	Guest Researcher	LMB NCI
C. Whitfield	Guest Researcher	LMB NCI

Project Description

Objectives:

To determine by genetic manipulation of CHO cells the cell proteins and processes needed to maintain the cytoskeleton and mitotic spindle; to analyze the molecular basis of the cellular response to cAMP, and the mechanism of transformation by Rous sarcoma virus.

Methods Employed:

Cell culture, virus infections, isolation of cell behavior mutants, karyotyping and G-banding, somatic cell hybridization, gene transfer using DNA and chromosomes, recombinant DNA techniques, immunologic techniques including immunoprecipitation, immunolocalization and use of monoclonal antibodies, one- and two-dimensional electrophoretic and chromatographic analysis of DNA, RNA and proteins; enzymologic analyses.

Major Findings:

(1) We have continued to analyze temperature-sensitive mutants with altered α- and β-tubulins. These mutants have normal interphase microtubules but cannot make normal spindles at the non-permissive temperature. Both abnormal spindles and apparently normal cytoplasm microtubules when isolated biochemically are found to contain both mutant and wild-type tubulins, indicating that the mutations are not affecting unique species of spindle-specific tubulins. Although the β-tubulin genes comprise a multigene family which consists of at least a dozen genes and pseudogenes recognizable on Southern blots, DNA mediated transfer of the Colcemid-resistance phenotype of one of the mutants has allowed us to identify the single β-tubulin gene which is responsible for drug-resistance and the spindle defect. This gene is amplified in a drug-resistant transformant resulting in overexpression of the mutant β-tubulin and Colcemid-dependence at 37°.

(2) Our β-tubulin mutants and transferents are resistant to the drug DCBT (dichlorobenzylthiocyanate), proving that this drug exerts its toxic effects on cells by interacting with microtubules. This drug is an unusual anti-microtubule agent since its inhibition of microtubule depolymerization requires pre-incubation.

(3) In an effort to understand the pleiotropic effects of microtubule depolymerization on cell function, we have studied the mobility of spin label probes in the plasma membrane of CHO cells treated with either colchicine, Colcemid, vincristine or griseofulvin. In all cases, these agents increase plasma membrane fluidity. These effects are blocked in our β-tubulin mutants, indicating that they are mediated through microtubule depolymerization.

(4) We have been studying S49 mouse lymphoma mutants in order to understand the role of cell adhesion in determining tumorigenicity. For fibroblast systems, decreased adhesiveness correlates with increased tumorigenicity. The nonadherent S49 mouse lymphoma line is highly tumorigenic; however, variants with increased adhesion have lost their tumorigenicity. These adherent variants (adh^+) express new cell surface antigens whose appearance correlates with immune rejection of the cells by the syngeneic host mice. This appears to represent an example of xenogenization, or the enhancement of immunogenicity of a cell line to decrease its tumorigenicity.

(5) Some cell types, such as epidermal cells, mammary epithelial cells and melanocytes require cAMP for growth, whereas others, such as lymphoid cells and fibroblasts (i.e., CHO cells) are inhibited by cAMP. We have found that when CHO cells are transformed by Rous sarcoma virus (CHO-RSV), they make tumors in nude mice only after treatment with cholera toxin which raises intracellular cAMP levels. Wild-type CHO cells are less tumorigenic after cholera toxin treatment. An increase in tumorigenicity is also seen in several lines of human melanomas after cholera toxin treatment. These results suggest that the oncogene which controls cell growth determines whether cAMP will stimulate or inhibit growth of tumors.

(6) Since most of the cAMP dependent protein kinase mutants carry dominant mutations encoding resistance to cAMP, it has been possible to transfer the cAMP-resistance phenotype from several mutants to sensitive cells via DNA mediated gene transfer. These transfers have been achieved using a two-step protocol. In the first step, genomic DNA is mixed with $_p$SV$_2$neo DNA and cotransferents are selected using the neomycin analog G418. In the second step these G418 resistant cells are selected for cAMP-resistance. Two of these transferents from different cAMP-resistant mutants have been shown to express the biochemical phenotype of the original mutants. In one case, using a cDNA probe for the regulatory subunit (R) of cAMP dependent protein kinase (obtained from S. McKnight) it has been possible to demonstrate DNA-mediated transfer of a mutant R-subunit gene. We have prepared cosmid libraries with DNA from two of our dominant mutants and are cloning the mutant protein kinase subunits from these libraries.

(7) Mutants with altered Type II cAMP dependent protein kinase have been found to be super-sensitive to the microtubule depolymerizing drugs Colcemid, colchicine and geldanomycin. This result suggests that cAMP dependent protein kinase interacts with the microtubule system in CHO cells to stabilize it. We have used this sensitivity to Colcemid and geldanomycin to select revertants with normal resistance to these drugs which have simultaneously acquired wild-type cAMP dependent protein kinase activity.

Significance to Cancer Research and the Program of the Institute:
National Cancer Plan Objective 3, Approach 3.5

CHO cells will cause tumors in appropriate hosts. The identification of mutant CHO cells with specific defects in regulation of cellular growth and morphology will enable us to determine whether any of these functions are needed for tumor formation. Once this information is obtained, specific therapy aimed at tumors neutralizing those growth promoting or cytoskeletal functions required for tumor formation can be devised.

Proposed Course:

We plan to continue to isolate many classes of mutants with abnormal cell behavior and determine specific protein alterations in these mutants. We will continue to study mutants we have already isolated: spindles and centrioles from tubulin mutant and revertant clones will be analyzed biochemically, the mechanism of these tubulin alterations at the DNA level will be determined, and altered genes involved in the anti-microtubule drug resistant and cAMP-resistant mutants will be cloned by gene transfer techniques from cosmid libraries.

Publications:

Gottesman, M.M., Roth, C., Vlahakis, G., and Pastan, I.: Cholera Toxin Stimulates Tumor Formation by Rous Sarcoma Transformed Cells. Mol. Cell. Biol. 4: 2639-2642, 1984.

Hochman, J., Levy, E., Mador, N., Gottesman, M.M., Shearer, G.M., and Okon, E.: Cell adhesiveness is related to tumorigenicity in malignant lymphoid cells. J. Cell Biol. 99: 1282-1288, 1984.

Gottesman, M.M., Vlahakis, G., Roth, C.: Genetic analysis of cAMP response in cultured cells. In Bolis, L., Verna, R., and Frati, L. (Eds.): Peptide Hormones, Biomembranes and Cell Growth. New York, Plenum Press, 1984, pp. 143-154.

Cabral, F., Schibler, M., Abraham, K., Whitfield, C., Kuriyama, R., McClurkin, C., Mackensen, S., and Gottesman, M.M.: Genetic analysis of microtubule function in CHO cells. In Borisy, G., Cleveland, D., and Murphy, D. (Eds.): Molecular Biology of the Cytoskelton. New York, Cold Spring Harbor Laboratories, 1985, pp. 305-317.

Gottesman, M.M.: Growth properties of Chinese hamster ovary cells. In Gottesman, M.M. (Ed.): Molecular Cell Genetics: The Chinese Hamster Cell. New York, Wiley-Interscience, Inc., 1985, pp. 130-154.

Gottesman, M.M.: Genetic analysis of cAMP dependent protein kinases. In Gottesman, M.M. (Ed.): Molecular Cell Genetics: The Chinese Hamster Cell. New York, Wiley-Interscience, Inc., 1985, pp. 711-743.

Gottesman, M.M.: Lineages of Chinese Hamster Cell Lines. In Gottesman, M.M. (Ed.): Molecular Cell Genetics: The Chinese Hamster Cell. New York, Wiley-Interscience, Inc., 1985, pp. 883-885.

Gottesman, M.M.: Chinese Hamster Cell Mutants. In Gottesman, M.M. (Ed.): Molecular Cell Genetics: The Chinese Hamster Cell. New York, Wiley Interscience, Inc., 1985, pp. 887-903.

Aszalos, A., Yang, G., and Gottesman, M.M.: Depolymerization of Microtubules Increases the Motional Freedom of Molecular Probes in Cellular Membranes, J. Cell Biol. 100: 1357-1362, 1985.

Singh, T.J., Hochman, J., Verna, R., Chapman, M., Abraham, I., Pastan, I.H., and Gottesman, M.M.: Characterization of a cyclic AMP resistant Chinese hamster ovary cell mutant containing both wild-type and mutant species of type I regulatory subunit of cyclic AMP-dependent protein kinase, J. Biol. Chem. In press.

Abraham, I., Brill, S., Hyde, J., Fleischmann, R., Chapman, M., and Gottesman, M.M: DNA-mediated gene transfer of a mutant regulatory subunit of cAMP-dependent protein kinase, J. Biol. Chem. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08706-14 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*

Alteration in Gene Expression During Mammary Gland Tumorigenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: G. H. Smith Research Biologist LMB NCI

COOPERATING UNITS *(if any)*

Department of Cell Biology, Baylor College of Medicine, Houston, Texas
Department of Pathology, Univ. of California, School of Medicine, Davis, CA

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have been studying alterations in expression of MMTV proviral and differentiation-specific genes such as casein and α-lactalbumin at different points in mammary gland tumorigenesis in a "clean" inbred mouse strain (C3H/Sm). This strain is of interest because "normal" expression of MMTV RNA transcripts in mammary gland is not accompanied by the appearance of viral structural proteins or by virions. Nevertheless mammary tumorigenesis, either experimentally induced or spontaneous, results in a significant increase in the abundance of these MMTV transcripts, especially a 2.2 Kb RNA containing only MMTV long terminal repeat (LTR) sequences. This transformation-related increase in abundance of MMTV LTR RNA has been extended to include preneoplastic mammary lesions (hyperplastic alveolar nodules) in C3H/Sm mice as well. Outgrowth lines of these preneoplastic lesions possess enhanced activities of their casein and α-lactalbumin genes in the absence of hormonal stimulation and release humoral factors which profoundly affect the development and differentiation of normal mammary tissue in mammary fat pads distal to their location in the host. We have tentatively identified several proteins encoded by MMTV LTR RNA which are present in these tumors and are abundantly represented in purified preparations of MMTV preprocapsids (intracytoplasmic A particles). These proteins bind strongly to nucleic acids and have the ability to unwind native double-stranded DNA. Competition studies indicate that these LTR ORF proteins show definite specificity for binding to LTR DNA sequences. Studies are in progress to determine whether these proteins have trans-acting potential for gene expression.

PHS 6040 (Rev. 1/84) GPO 914-916

Project Description

Objectives:

The aim of the project is to elucidate the genetic events in the mouse mammary gland leading to epithelial cell hyperplasia and eventually to neoplasia. Multiple factors, including virus, chemicals, hormonal stimulation of the gland and genetic susceptibility, have been identified as playing important roles in the development of mammary cancer. Therefore, our approach includes a multidisciplinary analysis of the changes in gene expression associated with mammary gland differentiation and development. Our objective is to identify and isolate genes or gene activities which may be important in the proliferation and maintenance of the neoplastic phenotype in mammary epithelium. We are presently examining mammary alveolar hyperplasias arising under the influence of a variety of oncogens (virus, chemical, hormone) and tumors which develop from these lesions. We are taking special interest in genes which become activated during growth and differentiation of normal mammary epithelium which are also expressed in transformed epithelium. Special emphasis will be given to the physiological state of the epithelial tissue in relation to its repsonse to a given carcinogenic agent. We have concentrated our efforts upon a unique mouse model for experimental breast cancer wherein we can compare and contrast preneoplastic lesions which arise through distinctly different morphogenetic pathways. Since these studies on breast cancer are carried out in the absence of overt retroviral involvement, they give promise of greater relevancy to the human disease.

Major Findings:

Mouse mammary tumor virus (MMTV) causes transformation of mammary epithelial cells, leading to carcinoma formation. However, at this writing no "transforming gene" analogous to those found in acutely transforming retroviruses has been identified in MMTV. Instead MMTV is thought to transform mammary cells by insertion of its DNA into host cell DNA and subsequent promotion of expression of an, as yet unidentified, mammary specific oncogene. The long terminal repeat (LTR) of MMTV has been shown to be a powerful promoter of eucaryotic gene expression from both *cis* and *trans* positions.

In our experiments, a 2.2 Kb MMTV LTR-containing RNA is increased in abundance in chemically, hormonally, or spontaneously transformed mammary epithelium, both preneoplastic and neoplastic. This increase is especially pronounced in mammary adenocarcinomas originating from ductal hyperplasias. The enrichment of this specific proviral transcript occurs in the absence of MMTV structural protein synthesis and without acquisition of new viral DNA by the transformed cell genome. Corticosteroid regulation of the LTR transcript *in vivo* varies directly with the growth and maintenance of the transformed mammary tissue. A cDNA library has been constructed from spontaneous mammary tumor RNA (C37-1), since the LTR-containing transcript is the major proviral transcript in this RNA, success in obtaining a cDNA LTR clone is greatly enhanced. In addition, the construction of genomic libraries from C37-1 and normal mammary gland DNAs

in cosmid vectors has been completed. The isolated recombinants will be used to characterize the flanking regions of the transformation-activated C3H/Sm proviral LTR gene(s) in an effort to identify potential mammary-specific oncogenes.

With polyclonal antibodies raised against synthetic peptides derived from the nucleotide sequence of the MMTV LTR open reading frame (ORF) (kindly provided by Dr. R. D. Cardiff, University of California, Davis), we have probed mammary tumor extracts and proteins synthesized in cell-free translation systems by polyadenylated tumor RNA for the translation products of our 2.2 Kb transformation-sensitive MMTV LTR RNA transcript. We have identified six LTR ORF-related proteins tightly associated with MMTV preprocapsids purified from MMTV-infected tumors. These proteins have the following approximate molecular weights, 36 Kd, 32 Kd, 18 Kd, 17 Kd, 16 Kd, and 12 Kd. Each of these LTR ORF-related proteins binds strongly to DNA. Three of these proteins, 36, 32 and 18 Kd, show DNA unwinding activity. In competition DNA binding experiments, these LTR-related proteins, especially the 18 and 16 Kd species, demonstrated substantial preference for binding to MMTV LTR DNA suggesting that they may play a regulatory role in MMTV gene expression or alternatively function to maintain free ends in MMTV DNA molecules thereby aiding recombination between viral and host DNA. These MMTV pronucleocapsid-associated proteins are strong candidates to represent the functional products of the MMTV LTR ORF. They possess biochemical features, e.g., the ability to bind and to destabilize DNA which suggest that they could implement the enhancing effect of the MMTV LTR on gene transcription. These observations provide a basis for clarification of the role of the LTR ORF in MMTV replication and tumorigenesis. Until now, the MMTV LTR protein, although predicted from the nucleotide sequence of the LTR open reading frame, had not been identified in vivo.

Eleven preneoplastic(immortalized) mammary explant lines were established in virgin hosts by serial transplantation into gland-free mammary fat pads. Eight of these preneoplastic lines display lobulo-aveolar hyperplasia reminiscent of mammary tissue in pregnant females. Each of these lines constitutively expressed mammary specific functional differentiation, i.e., synthesis of casein and α-lactalbumin in the absence of lactogenic hormonal stimulation. Three preneoplastic lines were established from mammary ductal hyperplasias found in urethane-treated females. These lines exhibit the mammary architecture of normal virgin mouse tissue with slightly enlarged ducts and ductules. These "ductal" hyperplasias did not constitutively express casein and α-lactalbumin in vivo but when placed in explant culture in the presence of lactogenic hormones showed morphological and functional differentiation to the same extent as normal virgin mouse mammary gland under similar culture conditions. All of these lines have MMTV LTR RNA transcripts at levels elevated above normal gland. In addition, we have found that growth, development and gene expression in normal mammary epithelium in fat pads distal to those carrying the preneoplastic outgrowths are modulated by the presence of the preneoplastic transplants. These observations suggest that preneoplastically transformed mammary tissue releases a humoral factor recognized by normal mammary epithelium. We are currently testing this hypothesis experimentally.

Significance to Cancer Research and the Program of the Institute:

National Cancer Plan Objective 3, Approach 3.

Mouse mammary gland tumorigenesis is a relevant experimental model for understanding human mammary neoplasia. The project is designed to provide a scientific basis to further our understanding of cellular and genetic events controlling normal development and differentiation and their relationship to malignant transformation of the mammary gland.

Proposed Course:

We propose to define the tumorigenic influence of endogenous retroviral (MMTV) genes and their possible relationship to normal cellular differentiation. This will be accomplished by studying the regulation of expression of differentiation-specific genes in mammary epithelium in relationship to the expression of MMTV proviral sequences. These events will be examined over the course of malignant transformation by comparing the regulation of these in normal, preneoplastic and malignant mammary tissues.

Publications:

Smith, G.H., Vonderhaar, B.K., Graham, D.E., and Medina, D.: Expression of pregnancy-specific genes in preneoplastic mouse mammary tissue from virgin mice. Cancer Research 44: 3426-3437, 1984.

Kidwell, W.R., Bano, M., Vonderhaar, B.K., and Smith, G.H.: Substratum modulates nutrient requirements and growth factor dependency of normal mammary cells in culture. In Barnes, D., Mather, J. and Sato, G. (Eds.): Growth of Cells in Defined Environments. Japan, Science Soc. Press. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08709-10 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Gene Expression and Differentiation by ADP-ribosylation of Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. S. Johnson	Research Chemist	LMB, NCI
OTHER:	R. Ralhan	Visiting Fellow	LMB, NCI
	C. Pribluda	Guest Researcher	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	2.4	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Drugs which inhibit $(ADP\text{-}ribose)_n$ synthetase and decrease endogenous ADP-ribosylation of chromosomal proteins cause accumulation of mRNA for glucocorticoid sensitive genes. Considerable variations in the extent of accumulation have been observed during the course of this study. Thus, factors in addition to the total amount of nuclear $(ADP\text{-}ribose)_n$ are involved. Glucocorticoid agonists or partial agonists are more effective agonists in cells devoid of $(ADP\text{-}ribose)_n$. Interestingly, certain steroids which bind to the receptor but do not normally induce genes are also very good agonists in these cells. We conclude that ADP-ribosylation of some essential protein(s), possibly the steroid receptor, influences expression of steroid-sensitive genes.

Nicotinamide and its synthetic N'-methyl derivative induce maturation of cultured human promyelocytic leukemia HL60 cells. The actions of these compounds are synergistic with retinoic acid, another agent which induces maturation of these cells. N'-methylnicotinamide is converted into N'-methylnicotinamide adenine dinucleotide. This NAD analog may be the active intracellular compound in the cells.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

Expression of genes is not uniform but rather is variable depending upon the cell's need to regulate metabolic functions or its differentiation process. Alteration in the regulation of genes results in cancer or other cellular abnormalities. The promoter region of the gene is important for regulation. In this portion of the gene certain chromosomal proteins interact with specific DNA sequences to regulate or modulate expression. However, little is understood about the nature of these proteins or how they interact with DNA to affect expression. Covalent modification of proteins may cause conformational changes in proteins and thereby alter gene expression. One such modification of chromosomal proteins is ADP-ribosylation. It is the object of this project to evaluate functions for ADP-ribose in the regulation of specific genes.

Methods Employed:

Standard culture techniques will be used to grow cells. Thin layer chromatography, high performance liquid chromatography and two dimensional gel electrophoresis will be used to analyze metabolic components and proteins. Standard biochemical techniques will be used to measure NAD^+, RNA metabolism and protein synthesis. mRNA will be detected and quantitated by hybridization to specific cDNA sequences.

Major Findings:

(A) Steroids induce expression of specific genes. Binding of the steroid to a receptor and subsequent association of this complex with the promoter region of genes are essential; however, it is not clear how the binding to chromatin results in gene induction. In the present research project, we are developing the proposal that loss of $(ADP\text{-}ribose)_n$ from chromosomal proteins is an important step in the activation process.

Treatment of cultured mouse mammary tumor cells with inhibitors of $(ADP\text{-}ribose)_n$ synthetase to decrease endogenous ADP-ribosylation of chromosomal proteins results in an increased expression of mouse mammary tumor virus (MMTV), a glucocorticoid-sensitive genome which is commonly used as a model system to understand gene regulation. In our initial studies, the induction was about the same as with glucocorticoid treatment. More recently for unknown reasons much smaller increases in MMTV RNA have been observed. In cells treated with $(ADP\text{-}ribose)_n$ synthetase inhibitors, glucocorticoid agonists are more effective activators of MMTV expression than they are in untreated cultures. Other steroids which bind to the receptor but are normally poor inducers or have no agonist activity are very effective in the treated cells. More steroid-receptor complex is found nuclear associated in the treated cells suggesting that either the steroid receptor complex or some component which influences its binding to chromatin is affected by treatment.

The observed rise in MMTV RNA levels could be due to a stimulation of transcription or a change in RNA stability. To begin this analysis transcription extension or nuclear "run-off" experiments were done. Our results thus far clearly demonstrate that RNA initiation is increased, but it is likely that some effects on RNA stability are also present.

(B) Various cells can be induced to differentiate in cell culture. These cultured cells have been used as model systems to understand regulation of differentiation. The cell line we have used is the HL60 human promyelocytic leukemia which can be induced to differentiate into cells with features of mature neutrophils or macrophages depending upon the culture conditions. We have found that HL60 cells acquire properties of mature neutrophils when treated with nicotinamide or its synthetic derivative N'-methylnicotinamide, agents which decrease $(ADP\text{-ribose})_n$ on proteins. Retinoic acid also induces maturation of these cells. HL60 cells treated with this latter agent display increased neutrophil functions and also increased tissue transglutaminase and NAD glycohydrolase activities. The latter enzymatic activities are properties of mature macrophages. Nicotinamide and retinoic acid synergistically increase the neutrophil functions but not the macrophage functions.

Nicotinamide decreases $(ADP\text{-ribose})_n$ by direct inhibition of $(ADP\text{-ribose})_n$ synthetase. N'-methylnicotinamide, however, does not affect enzymatic activity yet it decreases endogenous ADP-ribosylation of proteins, and its effects on cells are identical or very similar to those of nicotinamide. We have found that N'-methylnicotinamide exchanges with the nicotinamide moeity of NAD to form N'-methylnicotinamide adenine dinucleotide via an enzymatic reaction catalyzed by NAD glycohydrolase. It possible that this NAD analog is the active compound in the cells.

Significance to Cancer Research and the Program of the Institute:

By understanding how metabolism and gene expression are regulated and by understanding differences in this regulation between normal and transformed cells, we may learn to control the growth and differentiation of certain cancer cells. The studies with nicotinamide and retinoic acid may form the basis for treatment of certain types of cancer.

Proposed Course:

Interaction of specific proteins with unique DNA sequences is essential for regulation of individual genes in procaryotes. The steroid-receptor protein complex appears to regulate expression of certain genes in eucaryotes by a similar mechanism, but due in part to the complexity of the eucaryotic chromosome, details of this regulatory mechanism are only poorly understood. Our studies have uncovered a unique aspect of this regulation, i.e., ADP-ribosylation of chromosomal proteins is important. We proposed the following course to learn more about the role of ADP-ribosylation in gene expression.

(A) Gene Expression

Nicotinamide and the related drugs used in our study clearly perturb some essential cellular function to alter gene expression. Our working hypothesis is that they alter gene expression by inhibiting ADP-ribosylation of specific proteins, but this has not yet been clearly demonstrated. We will work toward providing direct evidence for this proposal and in so doing determine which proteins are influential in regulation of gene expression.

(1) For ADP-ribose to be essential for regulation of the MMTV genome, ADP-ribosylated proteins must be associated with regulatory regions of the genome. We will use two-dimensional analysis of nucleosomes to show association of ADP-ribosylated proteins with nucleosomes containing MMTV regulatory sequences (within the LTR). A "Western blot" analysis of DNA binding proteins will be done by transferring proteins from acrylamide gels to nitrocellulose. DNA binding proteins will be determined by subsequent binding to [^{32}P] DNA. The latter protocol will demonstrate evidence relative to DNA binding abilities of individual proteins to unique DNA sequences.

(2) The increased glucocorticoid activation of the MMTV genome in cells treated with benzamide indicates a more effective steroid-receptor interaction with chromatin in nuclei depleted of ADP-ribosylated proteins. We will compare binding of the steroid-receptor complex with chromatin from control cells and benzamide treated cells to directly demonstrate this possibility. Also we will analyze in more detail the components necessary or inhibitory for this interaction. It is also possible that benzamide treatment may affect the steroid receptor and thereby increase receptor function. This possibility will be determined by analyzing the binding characteristics of steroid to the receptor and the physical characteristics the activated receptor-steroid complex.

(3) Activation of the glucocorticoid genes may be subsequent to activation of another gene. We will analyze proteins synthesized in response to drug treatments. Emphasis will be placed on synthesis of nuclear, chromatin associated proteins.

(4) Induction of MMTV proteins will be determined to ensure that the RNA synthesized is translated into protein.

(5) More detailed kinetic analysis will be done to determine relative effects of drug treatment on RNA synthesis initiation and RNA stability.

(B) Differentiation

(1) One approach to the treatment of cancers is the use of agents which induce maturation of cancer cells into well differentiated or benign non-malignant cells. Retinoic acid treatment of some tumors is being used in this context. Our results, in an experimental model system for human cancer, demonstrate that nicotinamide and its derivatives cause maturation of

cancer cells. Moreover, their actions are synergistic with retinoic acid. Current studies are under way to evaluate the possible use of nicotinamide and N'-methylnicotinamide as therapeutic agents. This possibility is intriguing in that nicotinamide is a natural vitamin and is less toxic than are other agents, even at quite large doses.

(2) Differentiation and malignancy may be causally related to expression of oncogenes. If so, maturation of HL60 cells induced by nicotinamide may indicate an involvement of ADP-ribosylation of chromosomal proteins in regulation of oncogenes. We will test the effects of drugs on the expression of the oncogenes known to be present in these cells. If promising, these studies will be extended to other transformed cell systems to learn more about malignancy and oncogene expression.

Publications:

Lucas, D.L., Tanuma, S.-I., Davies, P.J.A., Wright, D.G., and Johnson, G.S.: Maturation of human promyelocytic leukemia cells induced by nicotinamide: evidence of a regulatory role for ADP-ribosylation of chromosomal proteins. J. Cell. Physiol. 121: 334-340, 1984.

Tanuma, S.-I., Yagi, T., and Johnson, G.S.: Endogenous ADP ribosylation of high mobility group proteins 1 and 2 and histone H1 following DNA damage in intact cells. Arch. Biochem. Biophys. 237: 38-42, 1985.

Davies, P.J.A., Murtaugh, M.P., Moore, W.T., Jr., Johnson, G.S., and Lucas, D.: Retinoic acid-induced expression of tissue transglutaminase in human promyelocytic leukemia (HL-60) cells. J. Biol. Chem. 260: 5166-5174, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08710-09 LMB

PERIOD COVERED

October 1, 1984 to September 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA Replication In Vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S. Wickner Research Chemist LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The molecular mechanisms involved in DNA replication are being studied biochemically. The system used is the in vitro initiation of replication of double-stranded λ dv plasmid DNA. This reaction requires two phage initiation proteins, O and P gene products, many host replication proteins, including the products of dnaB, dnaG, polC, dnaN, dnaJ, dnaK, dnaZ, dnaX, dnaY, gyrA, gyrB, and lig, RNA transcription in the region of the origin of replication and a specific site, ori, on the λ DNA. I have been isolating the proteins required for this reaction with the goal of reconstituting λ replication with all purified proteins. Towards this end, I have already prepared λ O, λ P, dnaB, dnaG, pol III holoenzyme (containing polC, dnaN, dnaX, and dnaZ polypeptides) and DNA gyrase. I have developed in vitro complementation assays for dnaJ and dnaK proteins and have designed purification procedures for their isolation. I am currently characterizing a weak ATPase activity associated with dnaK and DNA binding activity associated with dnaJ.

PHS 6040 (Rev. 1/84) GPO 914-916

Project Description

Objectives:

The object is to gain information about basic biochemical mechanisms involved in DNA replication by purifying and analyzing the proteins required for this reaction and reconstituting the pathways of replication with purified components.

Methods Employed:

Standard microbial genetic and biochemical techniques required for protein purification, enzyme assays, plasmid construction and nucleic acid sequencing are being used.

Major Findings:

I have been studying two E. coli proteins, dnaJ and dnaK gene products. These two proteins are required for both E. coli and λ DNA replication and are induced during heat shock. At this time their role in replication is not known. I have developed in vitro complementation assays for both dnaJ and dnaK in which crude extracts of either dnaJ - or dnaK - cells, when supplemented with purified λ O and P proteins, catalyze λ dv plasmid DNA synthesis upon the addition of dnaJ protein or dnaK protein, respectively. I have purified dnaJ protein to near homogeneity by eluting it from membrane pellets and subjecting it to column chromatography on phosphocellulose, hydroxyapatite and phenyl sepharose. I am now studying the purified protein and have discovered a DNA binding activity. I have also purified the dnaK protein to near homogeneity from crude lysates by column chromatography on DEAE Sephacel and hydroxyapatite. The final preparation contains a weak DNA independent ATPase as reported recently by Zylicz et al. Genetic experiments have suggested that λ P protein interacts with both dnaJ and dnaK proteins. I have indirect experiments to support those results and am now trying to isolate protein complexes directly by HPLC and sedimentation gradient centrifugation. I am expecting that a thorough characterization of dnaJ and dnaK proteins will help explain their function in replication.

I have also been studying the two phage proteins required for λ replication, the O and P gene products. The O protein is of particular interest since it is a specific DNA binding protein recognizing a 18 base pair sequence that is repeated four times in the region of the λ origin of replication. In collaboraation with Ken Zahn and F. Blattner, we showed that the DNA binding domain of the protein resides in the amino-terminal portion of the O protein. This was shown from studies of the protein fragment purified from cells carrying a plasmid in which we had cloned the part of the O gene coding for the amino-terminal portion of the O protein. Unlike the intact O protein, this protein fragment does not form dimers. Also it does not form a protein complex with λ P protein using conditions in which the intact O protein does. To directly relate these two functions to the carboxy-terminal portion of the O protein, I am cloning the part of the O gene coding for the carboxy-terminal half of the O protein.

In collaboration with Douglas Olendorf, we are trying to find conditions for crystallization of the amino-terminal DNA binding portion of the O protein. Our aim is to learn about the structure of O protein in the presence and absence of DNA. We will also soon attempt to crystallize the intact O protein and the P protein.

In collaboration with Mark Dodson and Hatch Echols, we are looking by electronmicroscopy at the protein-DNA structures formed on λ ori DNA by λ O proteins alone and in combination with P, dnaB, dnaJ and dnaK proteins.

The current model of how these various replication proteins are functioning is still vague. Presumably, in an early step, the λ O protein binds to the λ origin of replication through its amino-terminus. The P protein is directed to the λ DNA through its interaction with the carboxy-terminal portion of O. dnaJ and dnaK proteins are also attracted to the assemblying replisome through their interactions with λ P. dnaB protein is transferred to the DNA by P in a final prepriming reaction. Once on the DNA, dnaB serves as a mobile promoter for primer synthesis by dnaG primase. Primer elongation is then catalyzed by DNA pol III holoenzyme. It is anticipated that by studying the individual proteins and their interactions with each other, DNA and nucleoside triphosphates, a clearer picture of replication will emerge.

Significance to Cancer Research and the Program of the Institute:

The central process of heredity and cell growth is the replication of the genetic material. Studies of this process in E. coli have been made possible by the availability of mutants defective in DNA synthesis and large amounts of bacteria necessary for biochemical studies. The understanding that this work is generating about the biochemical mechanisms of DNA replication in E. coli has and will continue to shed light on the nature of the same reactions in animal cells.

Proposed course of research:

I will continue studying biochemical mechanisms involved in DNA replication using purified proteins and defined DNA templates. In particular, I will (1) characterize dnaJ and dnaK proteins and search for their function in replication, (2) further characterize the functional domains of λ O protein, and (3) reconstitute the λ replication reaction with all purified proteins and begin looking for prepriming complexes and primer RNA.

Publications:

Wickner, S.: DNA-dependents ATPase activity associated with Phage P22 Gene 12 protein. J. Biol. Chem. 259: 14038-14043, 1984.

Wickner, S.: Aligonucleotide Synthesis by E. coli dnaG primase in conjunction with Phage P22 Gene 12 protein. J. Biol. Chem. 259: 14044-14047, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08712-10 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ira Pastan	Chief, Laboratory of Molecular Biology	NCI
OTHER:	M. Willingham	Chief, UCS	LMB, NCI
	N. Richert	Senior Investigator	LMB, NCI
	S.-y. Cheng	Senior Investigator	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A biochemical quantitative method to follow ligands and receptors through clathrin-coated regions of the cell has been developed. These studies implicate coated pits in the Golgi region as having a crucial role in sorting and directing ligands and receptors to lysosomes. PMA induces the entry of the EGF receptor via coated pits but does not direct it to lysosomes. Instead, the receptor reappears on the surface after 30 minutes.

GPO 914-918

Other Professional Personnel:

J. Hanover	Postdoctoral Fellow	LMB, NCI
L. Beguinot	Guest Researcher	LMB, NCI

Project Description

Objectives:

To identify the membrane proteins that participate in endocytosis, exocytosis, and regulate cell metabolism and growth.

Methods Employed:

Cell culture, preparation and analysis of membrane lipids and proteins, subcellular fractionation, isolation of mutants with defective membrane proteins and defects in endocytosis.

Major Findings:

The organelles involved in intracellular sorting have been studied using a new quantitative method in which coated membranes (in a vesicular form) are isolated from a homogenate using anti-clathrin antibody adsorbed on staph aureus. It was found that EGF induces the EGF Receptor (EGFR) to pass first through a population of surface coated pits, later through a population of Golgi coated pits and finally to lysosomes. Phorbol myristate acetate causes EGF to enter cells via the surface pits, but it is not found in Golgi pits or lysosomes. Instead it returns to the surface about 30 minutes after entry. Likewise, the transferrin receptor enters cells via surface coated pits, and returns to the surface without passing through Golgi coated pits. These studies indicate that the coated pits of the Golgi have an important role in directing ligands and receptors to lysosomes.

The structure of coated pits involved in endocytosis has been studied by electron microscopy. During the endocytic process, pits go from shallow depressions on the surface to structures in which the coated pit is connected to the surface by a long narrow and often convoluted neck of uncoated membrane. The endocytic vesicle appears to form from the coated pit deep in the cytoplasm.

In collaboration with S.-y. Cheng, a membrane-associated thyroid hormone binding protein has been identified and purified and antibodies to it have been prepared.

Proposed Course:

A biochemical method is being used to quantify the transit of EGF, transferrin and their receptors through intracellular compartments containing clathrin-coated structures. Monoclonal antibodies, possibly directed to the α_2M receptor, are being used in studies of α_2M endocytosis.

Publications:

Hanover, J.A., Willingham, M.C., and Pastan, Il: Receptor-mediated endocytosis of α_2M: Solubilization and partial purification of the fibroblast α_2M receptor. Annals of the N.Y. Acad. Sci. 410-423, 1984.

Willingham, M.C. and Pastan, I.: Endocytosis and exocytosis: current concepts of vesicle traffic in animal cells. Int. Rev. Cytol. 92: 51-92, 1984.

Willingham, M.C., Strader, C.D., Lefkowitz, R.J., and Pastan, I.: Morphologic demonstration of clathrin-coated pits in frog and turkey erythrocytes. Exp. Cell Res. 151: 573-577, 1984.

Willingham, M.C., Hanover, J.A., Dickson, R. B., and Pastan, I.: Morphologic characterization of the pathway of transferrin endocytosis and recycling in human KB cells. Proc. Natl. Acad. Sci., USA 81: 175-179, 1984.

Willingham, M.C. and Pastan, I.: Do coated vesicles exist? Trends in Biochem. 9: 93-94, 1984.

Hasumura, S., Rossi, B., Alderson, R., Pastan, I., and Cheng, S.-Y.: Antibodies against the plasma membrane 3,3',5-Triiodo-L-Thyronine binding protein of rat pituitary GH_3 cells: Partial characterization and cross-species immunoreactivity Biochem. and Biophys. Res. Commun. 124: 956-962, 1984.

Willingham, M.C. and Pastan, I.: Ultrastructural immunocytochemical localization of the transferrin receptor using a monoclonal antibody in human KB cells. J. Histochem. Cytochem. 33: 59-64, 1984.

Lyall, R.M., Pastan, I., Waterfield, M., and Willingham, M.C.: EGF induces receptor down-regulation with no receptor recycling in KB cells. J. Cell Physiol. 122: 166-170, 1985.

Hanover, J., Pastan, I., and Willingham, M.C.: Inhibition of phosphatidyl-choline synthesis does not alter uptake of transferrin by LM fibroblasts. Exp. Cell Res. 157: 276-281, 1985.

Pastan, I., Hanover, J., and Willingham, M.C.: The cellular entry of EGF and transferrin: a problem in intracellular sorting. In Current Topics in Cellular Regulation 26: 17-25, 1985.

Beguinot, L., Hanover, J.A., Ito. S., Richert, N.D., Willingham, M.C., and Pastan, I.: Phorbol esters induce transient internalization without degradation of unoccupied epidermal growth factors receptors. Proc. Natl. Acad. Sci. USA 2: 2774-2778, 1985.

Alderson, R., Pastan, K., and Cheng, S.-Y.: Characterization of the 3,3'5-Triiodo-L-Thyronine-binding site on plasma membranes from human placenta. Endocrinology 116: 2621-2630, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08714-08 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mode of Action of a Bacterial Function Involved in Cell Growth Control

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S. Gottesman Research Chemist LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have been studying the role that protein degradation plays in regulating cell growth control, through the study of mutants defective in ATP-dependent protein degradation. E. coli lon mutants are defective in cell division regulation after DNA damage, and we have previously demonstrated that this defect is due to stabilization of a highly unstable cell division inhibitor, the product of the SulA gene. lon mutants also overproduce capsular polysaccharide, and we have developed a system for the study of the regulation of the genes necessary for capsule synthesis (cps), using cps::lac operon fusions. We have isolated and mapped mutations in three genes which regulate capsule synthesis (rcsA, rcsB, and rcsC). All three regulatory genes have been cloned, and the protein products are being identified. Genetic experiments indicated the existence of a cascade of regulatory interactions to regulate the transcription from the cps structural genes; future work will allow the in vitro reconstruction of this regulatory cascade and identification of the precise role of lon in this system. Studies on cells devoid of lon activity demonstrate the existence of other ATP-dependent proteolysis systems in E. coli. We will develop genetic selections for mutations in these other protease genes, as well as the biochemical characterization of these activities. In addition, we have demonstrated the in vitro degradation of a natural Lon substrate, the lambda N protein. Further studies of this degradation process will allow an analysis of the characteristics of Lon proteolysis.

PHS 6040 (Rev. 1/84) GPO 914-918

Other Professional Personnel:

P. Trisler	Research Biologist	LMB, NCI
A. Torres-Cabassa	Staff Fellow	LMB, NCI
M. Maurizi	Expert	LMB, NCI
T. Klopotowski	Visiting Scientist	LMB, NCI
K. Quinlan-Walshe	Research Biologist	LMB, NCI
J. Brill	Summer Student	LMB, NCI

Project Description

Objectives:

We are interested in the role which proteolysis can play in regulating important cell timing functions. As an approach to this, we are investigating the effects on cell growth of a mutation in a gene coding for an ATP-dependent protease, and developing genetic and biochemical methods for studying other bacterial proteases.

Methods Employed:

Standard microbial genetic and biochemical techniques.

Major Findings:

(1) We have continued the characterization of mutations in genes involved in the regulation of capsular polysaccharide synthesis. rcsA (a positive regulator) has been cloned on both λ and plasmid vectors and extensively mutagenized by insertional mutagenesis. Genetic results suggest that rcsA may be the mediator of lon effects on capsule synthesis. We are carrying out experiments to identify the rcsA product, and determine if it might be an unstable substrate of the lon system.

(2) Two other regulators of capsule synthesis, rcsB (a positive regulator) and rcsC (a negative regulator) have been cloned on λ vectors. Restriction maps and complementation experiments indicate that rcsB and C are separate loci, about 3 kb apart on the E. coli map. Insertional mutagenesis of these genes carried on a phage has allowed the identification of the rcsB product as a stable, 24 kd protein. rcsC is not easily visible under conditions where rcsB is abundant. Insertions in rcsB remain $rcsC^+$ and vice versa; therefore, these two genes are not in a single operon. Recent genetic experiments suggest the possibility that an additional regulatory gene or site (rcsD?) maps in this region.

(3) lon::Tn10 and lon deletion mutations, constructed by manipulation of cloned copies of lon and then transferred into the chromosome, have been shown to have all the properties of classical lon mutants but to still retain some ATP-dependent proteolysis. Monitoring of ATP-dependent proteolysis activity with anti-Lon antibody has demonstrated that this activity does not copurify with the small fragment of Lon made in these mutant cells. Therefore, lon

is not the only ATP-dependent proteolysis system in E. coli. This result is further confirmed by in vivo tests of proteolysis: starvation induced proteolysis is unaffected by these lon mutants, and degradation of some abnormal proteins is decreased in the lon mutant strains, but still proceeds at significant rates.

(4) Cells carrying a multicopy lon plasmid overproduce Lon more than ten-fold, as assayed with anti-lon antibodies. These cells grow poorly at low temperatures, accumulate insertions in the lon gene, and die at high temperatures. We are analyzing the defect in growth in these strains, in the expectation that high levels of lon may be causing the excessive degradation of essential E. coli proteins. In addition, we have shown that the high level of lon is sufficient to totally turn-off capsular polysaccharide synthesis, in agreement with our model that lon regulates capsule synthesis by degrading a positive regulator of capsule synthesis, the rcsA product.

(5) We have previously shown that λ N protein is highly unstable, and is stabilized functionally and chemically in lon mutants. Using purified N and purified lon, we have now demonstrated the ATP-dependent degradation of N by lon. This is the first example of the degradation in vitro of a natural lon substrate. While Lon eventually degrades N into a variety of small fragments, preliminary experiments indicate that there are a few preferential sites for the cleavage of N. Since the sequence of N is known, analysis of the cleavage sites will allow us to draw conclusions about the specificity of lon proteolysis. In addition, we are currently evaluating various approaches to a mutational analysis of the degradation process--i.e., the isolation of mutations in N which change its susceptibility as a Lon substrate.

Significance to Cancer Research and the Program of the Institute:

An understanding of the growth control of E. coli may serve as a model for understanding growth control in normal and transformed mammalian cells. Protein degradation is believed to play an important role in the control of mammalian cells; insight into such processes should be gained by further investigation of the process in E. coli.

Proposed Course of Research:

Elucidate the mechanism of regulation of capsular polysaccharide synthesis, at the genetic and biochemical level. Analyze the mechanism of Lon degradation of cellular substrates and the reasons for protein instability, using Lon substrates such as N as model systems. Use the methods developed in the study of the Lon substrates to genetically define other E. coli proteolytic systems and their substrates. Begin a study of the mechanism of cell division regulation, using the cell division inhibitor, SulA, as a probe which will specifically disrupt cell division.

Publications:

Trisler, P. and Gottesman, S.: lon transcriptional regulation of genes necessary for capsular polysaccharide synthesis in Escherichia coli K-12. J. Bacteriol. 160: 184-191, 1984.

Gottesman, S., Trisler, P., and Torres-Cabassa, A.: Regulation of capsular polysaccharide synthesis in Escherichia coli K-12: Characterization of three regulatory genes. J. Bacteriol. 162: 1111-1119, 1985.

Gottesman, S., Trisler, P., Torres-Cabassa, A., and Maurizi, M.R.: Regulation via proteolysis: The E. coli lon system. Microbiology. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08715-07 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Control of Synthesis of a Transformation-Dependent Secreted Glycoprotein

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M. M. Gottesman Chief, Molecular Cell Genetics Section LMB NCI

COOPERATING UNITS *(if any)*

Laboratory of Experimental Pathology, DCCP, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cultured mouse fibroblasts which are transformed by RNA viruses, a DNA virus or a chemical agent, all secrete a 39,000 Mr-phosphoglycoprotein (major excreted protein, MEP) in large amounts. Nontransformed murine fibroblasts secrete MEP after treatment with tumor promoters such as TPA or growth factors such as PDGF. Secreted MEP appears to be the precursor form of an acid protease with broad specificity. We have purified MEP, prepared monospecific affinity-purified antisera against it and cloned a cDNA which codes for MEP from Chinese hamster and mouse cells. The protein contains mannose 6-phosphate, the lysosomal recognition marker. It is processed intracellularly in both transformed and nontransformed cells to give two specific lower molecular weight forms, the lowest of which has a predominantly lysosomal localization. Transformation, TPA and PDGF stimulate MEP synthesis by increasing levels of MEP specific mRNA. The mechanism of the increase in MEP mRNA levels is increased transcription as measured in nuclear run-off experiments. We are studying this system as a model of regulation of lysosomal protease synthesis, processing and secretion as it is affected by transformation and agents which mimic the transformed state, such as tumor promoters and growth factors.

PHS 6040 (Rev. 1/84) GPO 914-918

Other Professional Personnel:

G. Vlahakis	Research Biologist	LMB NCI
M. Chapman	Research Biologist	LMB NCI
S. Gal	Chemist	LMB NCI
B. Troen	Medical Staff Fellow	LMB NCI
M. Rabin	Medical Officer	NIAID OD

Project Description

Objectives:

To determine the mechanism of the control of synthesis, processing and secretion of the major excreted protein of murine fibroblasts.

Methods Employed:

Cell culture; radiolabeling of cell proteins, electrophoretic and chromatographic techniques; immunoprecipitation; in vitro translation of mRNAs; recombinant DNA technology including full-length cDNA cloning, genomic cloning from cosmid libraries, hybrid selection techniques and Southern and Northern blots.

Major Findings:

(1) Synthesis and secretion of MEP (major excreted protein of transformed mouse cells) is increased in all transformed murine fibroblasts and is well-correlated with anchorage independence, and hence with tumorigenicity. Rat and hamster cell lines synthesize a similar protein which cross-reacts with MEP. Cultured human melanocytes and HeLa cells produce a small amount of protein which crossreacts with anti-MEP antibody. Synthesis of MEP is stimulated by treatment of cells with the tumor promoter, TPA, and the growth factor, PDGF.

(2) MEP is probably a lysosomal protein since it carries the mannose 6-phosphate lysosomal recognition marker, and is processed within cells to lower molecular weight forms of 29K and 20K which are localized to lysosomes. The purified 39K secreted form of MEP is stable at neutral pH, but between pH 3 and 4 it autodigests into lower molecular weight forms which have general endoprotease activity with a pH optimum of 3.5. MEP appears to be the precursor of a unique lysosomal acid protease because it is both inhibited by leupeptin and has a low pH optimum.

(3) We have cloned an almost full-length cDNA encoding mouse MEP. This probe recognizes a 1.8 kb mRNA in non-transformed and transformed mouse cells. The level of this MEP mRNA increases 5-10-fold after treatment of non-transformed cells with TPA or PDGF and approximately 30-50-fold in transformed cells. PDGF stimulation of MEP mRNA levels is blocked by cycloheximide and consequently, must require prior protein synthesis.

(4) In nuclear run-off experiments, transcription of MEP is dramatically increased by malignant transformation, TPA or PDGF. The half-life of MEP mRNA appears to be approximately 6-8 hours as determined in experiments using Actinomycin-D and determining MEP mRNA levels on Northern blots. This half-life is unaffected by transformation. Hence, the increase in MEP mRNA observed in transformed cells or cells treated with TPA or PDGF is apparently related primarily to increased transcription. These data indicate that expression of MEP is a good monitor of cellular response to transformation, TPA or PDGF.

(5) Southern blot analysis using mouse MEP cDNA probes suggests that there is only one mouse gene encoding MEP.

Significance to Cancer Research and the Program of the Institute:
National Cancer Plan Objective 3, Approach 3.5

MEP is both a marker of transformation and a sensitive indicator of the presence of at least one tumor promoter. The induction of its synthesis by tumor promoters could serve as a screening test for these agents in the environment. If MEP is found to fill an essential role in tumor growth or metastasis, then specific therapy aimed at neutralizing it could be designed as a model for cancer therapy.

Proposed Course:

To continue to analyze the molecular mechanism underlying induction of MEP by isolating genomic MEP clones in order to study the structure of the gene and the nature of its transformation-sensitive and growth factor responsive promoter; to construct MEP cDNA expression vectors for transfection of nontransformed cells and in vitro mutagenesis studies; to continue to use MEP as a marker of the molecular events involved in tumor promotion and transformation.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08717-07 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structural Analyses and Functions of Receptors for Cell Adhesion Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: K. M. Yamada Chief, Membrane Biochemistry Section LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Membrane Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.9	3.2	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cells appear to interact with structural and regulatory molecules in the extracellular matrix by means of specific receptors. We have analyzed putative receptors for two major glycoproteins, fibronectin and collagen. A membrane glycoprotein complex consisting of three components of approximately 140,000 daltons each was shown to co-localize partially with extracellular fibronectin and with intracellular α-actinin in microfilament bundles. Monoclonal antibodies against this complex blocked cell adhesion to fibronectin, and substrate-attached antibody alone could mimic fibronectin-mediated spreading. The components of this putative fibronectin receptor complex were isolated and shown to be three distinct acidic sialoglycoproteins associated noncovalently into an oligomeric complex. Other studies implicated gangliosides in cellular organization of extracellular fibronectin fibrils, and showed a localization of these lipids at sites of their attachment to the cell surface. Possible modulation of fibronectin receptor function by occupancy of the collagen receptor was explored. Incubation of cells with collagen or its α_1(I) chain was found to inhibit a specific subset of fibronectin receptor functions in non-competitive fashion. Cell spreading and phagycytosis were inhibited, while direct binding and cell attachment to fibronectin-coated substrates were unaffected. Our future objectives will be to analyze the structures and functions of the receptors for fibronectin and collagen. Monoclonal and polyclonal antibodies will be used with controlled proteolytic cleavage to define structural and functional domains of each. Alterations in their distribution and phosphorylation after transformation and heat shock will be established. Reconstitution experiments and analyses of other molecules needed for biological function should provide a further understanding of their mechanisms of action.

GPO 914-918

Other Professional Personnel:

K. Nagata	Visiting Associate	LMB, NCI
T. Hasegawa	Visiting Fellow/Guest Researcher	LMB, NCI
S. Saga	Visiting Fellow	LMB. NCI
H. Urushihara	Visiting Fellow	LMB, NCI
S. Yamada	Guest Researcher	LMB, NCI

Project Description

Objectives:

(1) To identify, localize, and analyze the biological functions of cell surface receptors for fibronectin and collagen.

(2) To determine the major structural features, functional organization, and mechanisms of action of these receptors using monoclonal antibodies, proteolytic dissection, and reconstitution experiments.

(3) To examine the regulation of fibronectin and collagen receptors, especially in neoplastic cells.

Methods Employed:

Rat hybridomas were generated after injection of Sprague-Dawley rats with preparations of the 140K membrane protein complex, followed by fusion of spleen cells with Y3 myeloma cells plated at cloning densities. High-affinity monoclonal antibodies were identified by screening for binding to a discrete antigen band in Western immunoblots after extensive washing. Hybridomas were adapted to grow in serum-free medium, and monoclonal antibodies were purified from culture supernatants by ammonium sulfate precipitation and DEAE chromatography.

To attach monoclonal antibodies to substrates, three methods were used. The monoclonal antibody was allowed to adsorb to tissue culture plastic wells, then excess antibody was washed away. Adsorbed antibody was then tested in biological assays. To prevent leaching from the substrate, parallel samples were cross-linked covalently to the plastic with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide. Alternatively, the antibody was covalently linked to the surface of a fixed gelatin film with 4% glutaraldehyde, followed by quenching with 3% BSA plus 0.1 M glycine. Spreading of cells on this and other ligands was quantitated by determining the percentage of cells with a complete circumferential zone of lamellar cytoplasm.

A localization procedure was developed for simultaneous correlative localization of an extracellular, a plasma membrane, and an intracellular antigen, as well as adhesion sites in the same cell, by combining interference reflection microscopy with triple-label immunofluorescence. Cells cultured on a glass coverslip were fixed, permeabilized with Triton X-100 and double-immunolabled with a mixture

of affinity-purified or monoclonal primary antibodies, followed by a mixture of rhodamine- and fluorescein-conjugated secondary antibodies that had been cross-adsorbed to remove reactivity with other antibodies in the mixtures. The coverslip was mounted in a filming chamber, and the cells were first photographed with an Antiflex objective by interference reflection microscopy; in this method, the regions of plasma membrane closest to the substrate appear black due to optical interference, and regions of intermediate distance appear grey. The objective was then changed to a standard objective for epifluorescence microscopy with the first two labels, as well as standard phase contrast microscopy. Several marked cells were then exposed to fluorescein excitation illumination to photobleach all of this bound fluorochrome. The cells were then labeled with a third antibody directly conjugated to fluorescein, and another photograph was taken. The 5 types of photograph of the same cell for each of the 3 immunological labels, phase contrast, and interference reflection microscopy could then be superimposed and compared.

The 140K protein complex was isolated with the monoclonal antibody JG22E. Crude membrane preparations from 13 day chick embryos were solubilized with 40 mM octylglucoside, then the protein was isolated using the monoclonal antibody immobilized on agarose. It was eluted with diethylamine and dialyzed against isotonic buffer containing octylglucoside.

Collagen was purified from pepsin-treated bovine collagen or rat tail collagen by salt fractionation. Although Lowry and absorbance measurements of protein concentration proved unreliable, the microbiuret method was found to accurately measure collagen concentrations. α_1(I) chains were isolated after heat-induced separation of collagen subunits and chromatography on CM cellulose at 43°C, followed by molecular sieve chromatography in 2 M guanidine-HCl and dialysis against acetic acid followed by adhesion medium. Effects of collagen on the binding of fibronectin and its 75,000 dalton fragment to BHK cells was evaluated using molecules labeled by reductive methylation with tritiated borohydride. Binding of substrate-adsorbed fibronectin and phagocytosis were measured with 0.8 micron diameter polystyrene beads coated with tritiated fibronectin; endocytosis was estimated by determining the amount of radioactivity becoming inaccessible to trypsinization. Cell attachment was determined on substrates coated with fibronectin, then counter-coated with 10 mg/ml heat-denatured BSA, in the presence and absence of inhibitor. After incubation, cells remaining attached after rotary agitation at 150 rpm were detached by trypsin and counted electronically to quantitate cell attachment.

Major Findings:

Normal and malignant cells interact with the extracellular matrix during cell adhesion, migration during embryonic development, tumor cell invasion, and metastasis. These interactions may be mediated by specific cell surface receptors, including specific, saturable receptors for fibronectin and collagen. A putative fibronectin receptor was found to consist of a set of 3 membrane glycoproteins of approximately 120,000, 135,000 and 155,000 daltons (termed 140K complex). In immunolocalization studies, monoclonal and polyclonal antibodies against members of this 140K membrane complex were found to co-localize in fibrillar patterns with fibronectin in adhesion sites of chick fibroblasts; this finding suggests a spacial association of the two proteins. In addition,

intracellular α-actinin in microfilament bundles was found to co-localize partially with anti-140K, particularly in adhesion sites identified by reflection interference microscopy. Functional relevance of the 140K complex to fibronectin-mediated adhesion was indicated by a marked inhibition of normal and transformed cell spreading on fibronectin after treatment with an anti-140K monoclonal antibody. Like fibronectin, these molecules that were inhibitory when in solution in excess became positive mediators of cell adhesion if adsorbed or cross-linked chemically to substrates. These findings are consistent with a possible function of the 140K complex as a part of a transmembrane linkage mechanism between cell surface fibronectin and intracellular microfilament bundles. Preliminary experiments indicated that levels of this complex were slightly increased after transformation by Rous sarcoma virus, and that its fibrillar organization was completely disrupted.

The 140K complex was characterized biochemically. It was found to consist of three polypeptides associated in a non-convalent oligomeric complex as determined by sucrose gradient centrifugation. The components were unrelated according to two-dimensional peptide mapping, indicating that they are distinct proteins rather than processed forms of a single protein. Each was found to exist as a set of regular isoelectric point variants with acidic pI's, and each was shown to contain sialic acid that accounted for the heterogeneity. Amino acid analysis showed an above-average content of cysteine, consistent with known alterations in the electrophoretic mobility of the 140K complex after chemical reduction. New monoclonal antibodies to this complex were obtained, and we are presently characterizing a library of 120 new monoclonal antibodies specific for bands of the complex. Preliminary studies indicate that the isolated complex may interact with the cell-binding region of fibronectin and laminin, as determined by affinity chromatography.

We have examined for other molecules necessary for receptor function. A mutant cell line developed by Moss and Fishman that lacks higher-order gangliosides was unable to organize fibronectin fibrils. Addition of exogenous fluorescently-labeled gangliosides resulted in reconstitution of fibril formation, and a partial co-localization of added gangliosides was found with the attachment sites of fibronectin to the plasma membrane. These findings implicate gangliosides in fibronectin fibril formation. Revertants and double revertants of these cells are now under investigation, as is the capacity of these variant cells to undergo adhesive interactions with substrate-bound fibronectin. In other ongoing studies, a particular lot of polyclonal antibody against mouse 3T3 cell membranes was found that was a specific inhibitor of fibronectin-mediated adhesion; antigen blocking experiments revealed two size classes of molecule necessary for interactions with fibronectin. One class corresponded to the 140K antigen, but a second class was a glycoprotein of 45,000 daltons. This molecule is being further characterized.

The possible regulation of fibronectin receptor function by other extracellular molecules was examined. Native type I collagen or its purified α_1(I) chain inhibited specific aspects of fibronectin receptor function. Spreading of cells on fibronectin-coated substrates and phagocytosis of fibronectin-coated beads were inhibited in a non-competitive manner. In contrast, the initial attachment of cells to fibronectin and the direct binding of fibronectin to its receptor

were unaffected. We hypothesize that binding of collagen by its high-affinity receptor modulates function of the fibronectin receptor at a step distal to the initial receptor binding step. In preliminary studies, characterization of a fibroblast receptor for collagen showed that it may be identical to a major transformation-sensitive membrane protein of unknown function previously studied by us and others. The loss of this receptor may account for known defects in the organization of extracellular collagen by transformed cells. Finally, other preliminary results suggest that this receptor is a heat-shock regulated protein that is differentially phosphorylated in transformed cells, which appears to be the first identification of a function and transformationrelated modification for any member of this well-studied regulatory system, whose proteins were of hitherto unknown function.

Significance to Cancer Research and the Program of the Institute:

Invasion and metastasis are hallmarks of malignant cells, yet relatively little is known about the biochemical mechanisms by which they interact with the molecules of the tissues they invade. Such interactions within extracellular spaces are likely to occur by plasma membrane receptors, especially those for fibronectin and collagen. Our studies are attempting to characterize the structures and functional mechanisms of these two receptors, as well as examining how they influence each other and are altered by transformation. Information on these two receptor systems and their regulation in normal and malignant cells should further our understanding of the metastatic process.

Proposed Course of Research:

We propose to extend our experiments on the receptors for fibronectin and collagen (1) to use proteases and other cleavage reagents to define structural and functional domains of each, (2) to characterize their alterations in transformed cells in terms of quantities, distribution, phosphorylation, and binding kinetics for their respective ligands, (3) to raise monoclonal antibodies to the collagen receptor for future tissue localization, structural, and DNA cloning experiments, (4) to characterize the 120-member monoclonal antibody library defining different epitopes of the 140K complex in terms of polypeptide binding site, intracellular or extracellular exposure, and localized inhibition of function, in order to dissect the mechanism of action of the 140K complex in cell interactions with fibronectin and laminin, (5) to attempt reconstitution of these putative receptors in model systems and into cells lacking them, (6) to characterize the regulation of these proteins by temperature, since the heat-shock system is considered a prototype transcriptional regulation system, and (7) to complete experiments examining the role of gangliosides and the 45,000 dalton membrane antigen in receptor interactions with fibronectin.

Publications:

Yamada, K.M. and Akiyama, S.K.: Interactions of cells with extracellular matrix components. In Elson, E.L., Frazier, W.A., and Glaser, L. (Eds.): Cell Membranes Vol. 2. New York, Plenum Publishing, Corp., 1984, pp. 77-148.

Yamada, K.M., Hasegawa, T., Hasegawa, E., Kennedy, D.W., Hirano, H., Hayashi, M., Akiyama, S.K., and Olden, K.: Fibronectin and interactions at the cell surface. In Hinchliffe, J.R. and Kemp, R.B. (Eds.): Matricies and Differentiation. New York, Alan R. Liss, Inc., 1984, pp. 1-15.

Chen, W.-T., Hasegawa, E., Hasegawa, T., Weinstock, C., and Yamada, K.M.: Development of cell surface linkage complexes in cultured fibroblasts. J. Cell. Biol. 100: 1103-1114, 1985.

Spiegel, S., Yamada, K.M., Hom, B.E., Moss, J., and Fishman, P.H.: Fluorescent gangliosides as probes for the retention and organization of fibronectin by ganglioside-deficient mouse cells. J. Cell. Biol. 100: 721-726, 1985.

Hasegawa, T., Hasegawa, E., Chen, W.-T., and Yamada, K.M.: Characterization of a membrane-associated glycoprotein complex implicated in cell adhesion to fibronectin. J. Cell. Biochem. In press.

Yamada, K.M., Akiyama, S.K., Hasegawa, T., Hasegawa, E., Humphries, M.J., Kennedy, D.W., Nagata, K., Urushihara, H., Olden, K., and Chen, W.T.: Recent advances in research on fibronectin and other cell attachment proteins. J. Cell. Biochem. In press.

Thiery, J.P., Boucaut, J.C., and Yamada, K.M.: Cell migration in the vertebrate embryo. In Edelman, G. (Ed.): Molecular Determinants of Animal Form. New York, Alan R. Liss, Inc. In press.

Nagata, K., Humphries, M.J., Olden, K., and Yamada, K.M.: Collagen can modulate cell interactions with fibronectin. J. Cell. Biol. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08719-06 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development and Uses of Eukaryotic Vectors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: B. H. Howard Chief, Molecular Genetics Section LMB, NCI

COOPERATING UNITS *(if any)*

LMV, DCE, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The primary focus of work with eukaryotic vectors has shifted during the past year to investigation of growth inhibitory activity detected in a DNA-mediated gene transfer system. Progress has been made in defining the range of cell types from which genomic DNA demonstrating strong growth inhibitory activity can be prepared. Molecular cloning of growth inhibitory sequences is being pursued by analysis of a cosmid library derived from WI38 human embryo fibroblast genomic DNA. Both serial gene transfer and fractionation ("sib selection") techniques have been employed, with the result that a single candidate cosmid clone exhibiting high growth inhibitory function was identified. Studies on growth-stimulatory genes concentrated on characterization of c-H-ras transformed rat embryo fibroblasts with respect to tumorigenicity and metastatic phenotype. Continuing efforts to improve gene transfer technology involved developmental experiments on vectors that code for novel cell surface antigens, further application of a vector system based on the bacteriophage lambda lysogenic cycle, and preparation of helper virus-free retrovirus/dihydrofolate reductase vector stocks for infection of mouse bone marrow cells.

PHS 6040 (Rev. 1/84) GPO 914-918

Other Professional Personnel:

R. Padmanabhan	Chemist	LMB, NCI
N.-Z. Xu	Visiting Fellow	LMB, NCI
M. M. McCormick	Guest Researcher	LMB, NCI
R. B. Stead	Medical Staff Fellow	LMB, NCI
T. Howard	Guest Researcher	LMB, NCI
I. Pastan	Chief, Laboratory of Molecular Biology	LMB, NCI
M. Willingham	Chief, Ultrastructural Biochemistry Section	LMB, NCI
M. Gottesman	Chief, Biochemical Genetics Section	LMB, NCI
G. Gaitonaris	Visiting Fellow	LMB, NCI
A. Singer	Senior Investigator	IB, NCI
D. Singer	Senior Investigator	IB, NCI
G. Schwartz	AFFRI	
R. Pozzatti	Guest Researcher	LMV, NCI
G. Khoury	Chief, Laboratory of Molecular Virology	NCI
R. Reeves	Professor of Biochemistry, Washington State U.	

Project Description

Objectives:

The objectives of this project are three-fold. First, we will continue systematic development of vectors and techniques to extend the range of mammalian cell types into which genes can be efficiently transferred. Second, we will place special emphasis on the use of this technology to identify and isolate genes and/or DNA sequences that regulate mammalian cell growth. Third, we will utilize gene transfer in conjunction with analysis of mRNA and protein levels to study mechanisms by which such growth regulatory genes exert their effects.

Methods Employed:

Vectors designed for amplification of genes in *E. coli* and subsequent transfer to mammalian cells are crucial to this area of research. In the pSV2 family of vectors the ampicillin resistance cistron and origin of replication from the plasmid pBR322 are used for selection and propagation in *E. coli*; simian virus 40 (SV40) early region promoter and mRNA processing signals are used to direct expression in mammalian cells of Tn*9* chloramphenicol acetyltransferase (cat), *E. coli* xanthine guanine phosphoribosyltransferase (gpt), or Tn*5* aminoglycoside phosphotransferase (neo) coding sequences. In the pRSV family of vectors the promoter in the long terminal repeat (LTR) of Rous sarcoma virus (RSV) is substituted for the SV40 early promoter to direct expression of cat, gpt, neo, or dihydrofolate reductase (dhfr) coding sequences. Retrovirus vectors are based on Moloney leukemia virus. Mouse methotrexate-resistant dihydrofolate reductase (dhfr) cDNA is inserted in place of the gag-pol coding region.

These vectors are most frequently introduced into mammalian cells using the calcium phosphate-DNA coprecipitation technique. Alternate methods involving

DEAE-dextran/DNA complexes and liposome fusion are investigated where appropriate. Retrovirus vectors introduce foreign genes by infection, with helper functions provided by coinfection with non-defective virus or by earlier propagation in specialized γ_2 non-producing helper cells.

Several assays for expression of these vectors in mammalian cells are available. Total transient expression (12-60 hrs after transfection) may be assayed, when cat vectors are used, by determining 14-C chloramphenicol acetylated by cat in cell extracts. With other vectors S1 nuclease or reverse transcriptase primer extension analyses can be used to determine steady-state mRNA levels. The percentage of cells expressing cat vector DNA may be assayed by detection of intracellular cat with rhodamine-labeled cat antibody or cat antibody in conjunction indirect rhodamine or peroxidase labeling. Both affinity-purified and monoclonal cat antibodies are available. The number of cells stably expressing vector genes may be assayed by colony selection using the gpt, neo, or dhfr markers. Colonies selected may be amplified and used for quantification of integrated exogenous DNA copies (Southern blots), mRNA (Northern or S1 nuclease analysis), and cat activity.

Finally, to isolate mammalian DNA sequences detected by these techniques, genomic libraries are constructed using either cosmid vectors or λSV2/cosmid vectors.

Major Findings:

I. Mammalian vector development

A. Vectors of the RSV vector family (pRSVneo, pRSVgpt, pRSVdhfr, and pRSVdhfr-m): These constructs were used in various projects described below without further modification.

B. Lambda.SV2 and derivative vectors: In conventional bacteriophage lambda and plasmid vectors, deletions and rearrangements of insert fragments are frequently observed. Deletions are particularly prone to occur in very large DNA inserts. To minimize these instability problems, lambda.SV2, a vector that integrates by site-specific recombination into the chromosome of the specialized lambda lysogen N6106, was previously developed. To facilitate uptake of recombinant molecules that carry inserts in the size range of 40-50 kilobase pairs, a cosmid derivative of lambda.SV2 was also constructed. Current work is primarily in following areas:

i) Improvement of vector replication following induction of the host temperature sensitive lysogen. The low yield of lambda.SV2 recombinant molecules has represented the main impediment to more widespread use of this system. Strategies to improve yields include: a) inversion of ampicillin and chloramphenicol markers (to eliminate transcription from these cistrons in the anti-sense direction into the lambda replication origin of the vector); b) incorporation of *in vivo* packaging functions into the host lysogenic strain (to permit recovery of molecules that replicate by the rolling circle mode); and c) insertion of the chi recombination sequence (to facilitate formation of dimers for *in vivo* packaging).

ii) Derivation of large inserts by in vivo recombination. Although in vitro packaging dramatically improves transfection efficiencies, it imposes a maximum size limit of 40-45 kilobase pairs on insert fragments. To circumvent this limitation, an approach was proposed in which the insert in an integrated lambda.SV2 molecule could be extended by homologous recombination with a partially overlapping fragment in a second lambda.SV2 vector. This approach was successfully tested by reconstitution of the histidine operon. (See the section submitted by Max Gottesman for details on this system.)

C. Retrovirus vectors: Vectors based on mammalian retroviruses offer the possibility of extremely efficient introduction of foreign sequences into many cell types. Although these vehicles are associated with novel limitations (e.g., incompatibility with multiple transcription and mRNA processing signals in insert sequences) they should complement standard DNA-mediated transfection techniques. We previously constructed a retrovirus vector that carries a mouse cDNA methotrexate-resistant dihydrofolate reductase gene. In the past year high titer helper virus-free stocks were generated and used in attempts to infect mouse bone marrow cells or preimplantation mouse embryos. The immediate goal of these experiments (carried out in collaboration with D. Singer and A. Singer) is to derive a mouse bone marrow population that exhibits increased resistance in vivo to methotrexate. It is hoped that methotrexate resistance could provide a selective propagation advantage to this population in mouse/ mouse or mouse/rat marrow chimeras treated with low doses of methotrexate. If a selective advantage can be demonstrated, then it may be possible to examine effect(s) of donor:recipient hematopoietic cell ratios on transplantation immunity.

D. Vectors that code for novel cell surface proteins (collaboration with Drs. Ira Pastan and Mark Willingham): Vectors that allow identification and/or physical separation of cells transiently expressing exogenous DNA would be extremely useful. Our immediate interest is in developing a more rapid assay for growth inhibitory sequences (see below). Further applications of such vectors could include: i) measuring alteration(s) in endogenous gene expression 12-48 hr following cotransfection with, for example, plasmids carrying putative growth regulatory genes; ii) modification of endogenous gene expression by cotransfection of anti-sense coding plasmids; and iii) isolation of genes from cosmid libraries by assaying for alterations in endogenous gene expression (as in "i") in conjunction with sib fractionation of cosmid pools. Candidate inserts for such cell surface protein vectors code for: vesicular stomatitis virus G protein, influenza hemagglutinin, Moloney leukemia virus envelope protein, Thy 1.1 protein.

II. New methods to improve DNA-mediated stable transformation efficiencies.

A. Effects of sodium butyrate (in collabroation in R. Reeves): Experiments were resumed in an effort to document whether increased expression of transfected plasmid DNA molecules could be correlated with an alteration in plasmid chromatin structure. Both increased plasmid DNase sensitivity and hyperacetylation of histones on acutely transfected molecules were documented.

B. Effects of SV40 T antigens: WI38 human embryo fibroblasts can be stably transfected by pRSVneo at a frequency of $1\text{-}2x10^5$. It was previously observed that this frequency could be increased 5-10 fold by cotransformation with either SV40 DNA or a non-replicating recombinant genome, pRSV-TAg, in which T antigen expression is directed by the RSV long terminal repeat (Xu Nai-Zheng and B. H., unpublished results). In this reporting period it was demonstrated that the enhanced stable transformation frequency could be completely accounted for by an elevation in transient expression (measured by activity of pRSVcat). To determine whether T antigen increases the fraction of cells competent to express exogenous DNA, staining of pRSVcat-transfected cell populations with affinity purified CAT antibody (provided by Dr. Ira Pastan) is being carried out.

C. Effects of altering cell morphology: Many cell types that grow in suspension are refractory to DNA-mediated gene transfer. An obvious, if somewhat naive, idea is that DNA uptake could be improved by the simple expedient of temporarily inducing a flattened "epithelioid" morphology. Two potentially complementary strategies are being investigated: i) increased cell-substrate interaction by the use of poly-lysine, cell surface specific antibodies, or concanavalin A, and ii) application of centrifugal force during exposure of cells to calcium phosphate-DNA coprecipitates.

III. Application of gene transfer methods to detection of genes that regulate cell growth.

A. Malignant transformation of rat embryo fibroblasts: (collaboration with Drs. R. Pozzatti and G. Khoury; NCI, DCE, LMV). Following negative results in experiments directed at malignant transformation of monkey kidney CV-1 cells, attention was turned to transformation of rat embryo fibroblasts (REF). In contrast to published results from the laboratory of Dr. R. Weinberg (MIT), it was found that primary REF could be stably transformed (criteria: growth in soft agar and formation of tumors in nude mice) by cotransfection with the c-H-ras gene and a selectable neo marker. Genes such as adenovirus E1A and c-myc enhanced the frequency of, but were not absolutely required for, complete malignant transformation. We were further surprised to find that c-H-ras transformed REF demonstrated high metastatic potential in a nude mouse system.

B. Inhibition of HeLa cell replication: In the previous annual report we described preliminary evidence that sequences present in WI38 human embryo fibroblasts are capable of slowing HeLa cell growth. Results were obtained from experiments in which HeLa cells in monolayer culture were transfected with pRSVneo DNA in combination with either E. coli, HeLa, or WI38 genomic DNA. Forty-eight hours after transfection, cells from each culture were transferred into suspension medium containing bromodeoxyuridine and Hoechst 33258. After 48 hrs in suspension, cells were exposed to fluorescent light for 5 minutes, then plated into G-418 medium. This protocol was designed to select cells that were prevented by exogenous DNA sequences from replicating in suspension culture and, in addition, stably expressed the pRSVneo selectable marker. We determined that transfection with WI38

DNA increased cell survival at least 10-fold more efficiently than either E. coli or HeLa S3 DNA. Moreover, the average growth rate of G-418 resistant cells following transfection with the the WI38/pRSVneo mixture was about 2-fold slower than controls. The major effort of this group over the past year has focused on confirming and extending these observations as follows:

i) Search for more rapid assays to detect growth inhibitory sequences: Although a "monolayer" version of the assay for growth inhibitory sequences generated data that has subsequently been confirmed by our standard assay, the "signal-to-noise" ratio was not satisfactory. We are therefore investigating alternative cell recipients in the hope that one of these will be more sensitive to growth inhibitory factors than is the HeLa cell line (and thus not require spinner culture conditions to exhibit substantial growth slowing). We are also attempting to develop an assay based on cotransfection with a cell surface protein expression vector, treatment with colchicine, and analytical cell sorting to determine the fraction of transfected cells that are temporarily fixed in the G0/G1 phase of the cell cycle.

ii) Genomic DNAs prepared from other sources have been examined for the presence of growth-inhibitory sequences. NIH/3T3 DNA was previously reported to be negative, indicating that not all fibroblast cell strains are active. DNAs from rat embryo fibroblasts and low (20-25th) passage MRC-5 human lung embryo fibroblasts are also negative. Surprisingly, genomic DNA from MRC-5 cells rendered quiescent by maintenance in 0.5% fetal calf serum is strongly positive. This result suggests that at least one major growth inhibitory sequence subset is controlled by epigenetic mechanism(s), e.g., DNA methylation or rearrangement. DNAs from VA-13 (a post-crisis SV40-transformed WI38 derivative), and T24 (a c-H-ras positive human tumor cell line), are positive, suggesting that alternative immortalization/transformation mechanisms exist which are consistent with constitutive expression of growth inhibitory genes. SIHA and Caskey, cell lines that (like HeLa) carry integrated copies of human papilloma virus, are currently being investigated.

iii) It was previously reported that Rev-2, a non-clonal WI38 DNA-transfected HeLa cell population, exhibits increased sensitivity to growth inhibition by low serum relative to parental HeLa cells. These cells were tested for tumorigenicity in the nude mouse system. Although delayed in growth was evident, a $2x10^5$ Rev-2 cell inoculum produced tumors in 4/5 animals (as opposed to tumors in 5/5 animals injected with this number of HeLa cells). No tumors formed in animals injected with smaller inocula of either Rev-2 or HeLa cells. Segregation of tumorigenic variants from the Rev-2 population in the absence of a selection for the G-418 resistance marker is an obvious potential problem. In such in vivo tumorigenicity experiments, it will probably be necessary to examine clonally derived WI38/pRSVneo-transfected cell populations with paired G-418 sensitive revertants as controls.

iv) Cosmid libraries containing E. coli, HeLa, or WI38 genomic sequences were previously constructed. The WI38 library exhibited a strong growth-inhibitory signal, suggesting that clone(s) carrying functional WI38 sequences of interest were present. The E. coli and HeLa libraries were 8- to 20-fold less active. In this reporting period, fractionation of the WI38 library was completed and sublibraries with complexities ranging from 70 to 70,000 colonies tested. All of these sublibraries were positive for growth inhibitory sequences, suggesting a copy number in excess of 10^3. Of four cosmids sampled from the 70 colony sublibrary to date, one is strongly positive. As before, it will be necessary to confirm that sequences detected in the WI38 library, sublibraries, and single cosmid clone are related to those active in WI38 genomic DNA.

v) Genomic DNA from Rev-2 cells was noted in the last report to carry growth-inhibitory sequences in secondary transfection experiments. Although DNA from Rev-2 secondary transfectants was also positive for growth inhibitory sequences, rescue of the ampicillin-resistance marker (carried in pRSVneo) from this DNA was unsuccessful. Primary and secondary transfections have now been carried out starting with the WI38 cosmid library. WI38 library secondary transfectants appear to carry cosmid sequences, criteria being slow growth in gpt selective medium and a positive dot blot for vector sequences. If the sublibrary fractionation strategy (iv) yields ambiguous results on further investigation, an attempt will be made to rescue cosmid sequences and linked DNA from these WI38 library secondary transfectants.

Significance for Cancer Research and the Program of the Institute:
National Cancer Plan Objective 6, Approach 3

DNA-mediated transfer of genetic material into mammalian cells is a powerful approach for identifying mechanisms that control cell growth and for elucidating how malfunction of those mechanisms leads to malignant transformation. The project described above is directed towards the systematic improvement of gene transfer technology , as well as towards its simultaneous application to identify genes and/or DNA sequences that regulate mammalian cell growth.

Proposed Course:

Further work will focus on refining mammalian vector systems, improving methods for introduction of DNA into mammalian cells, and understanding factors which control stable expression of exogenous genes. Emphasis will be placed on application of gene transfer techniques to primary cells such as murine hematopoietic stem cells and human embryo fibroblasts. Results on inhibition of HeLa cell replication will be extended. Initially, our efforts will concentrate on isolation of the DNA sequence(s) that mediate this experimental effect. If these sequences can be cloned, then we will investigate the relationships such sequences may have to malignant transformation, in vitro senescence, and differentiation.

Publications:

Hamada, H., Seidman, M., Howard, B.H., and Gorman, C.M.: Enhanced gene expression by the poly(dT-dG) · poly(dC-dA) sequence. Mol. Cell. Biol. 4: 2622-2630, 1984.

Howard, B.H. and McCormick, M.: Vector-mediated gene transfer. In Gottesman, M.M. (Ed.): Molecular Cell Genetics. New York, John Wiley and Sons, Inc., 1985, pp. 211-233.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08750-05 LMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic Regulatory Mechanisms in Escherichia Coli and Its Bacteriophage

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Adhya	Chief, Developmental Genetics Section	LMB, NCI
OTHER:	S. Garges	Microbiologist	LMB, NCI
	R. Haber	Biologist	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The cya, crp, rho and nus gene products modulate the expression of a wide variety of bacterial and bacteriophage genes or operons. In order to understand the regulatory processes, we are studying the structure, expression and activity of these genes. We have previously shown that the protein products of the cya, crp, and rho genes are autogenously regulated. In CRP, cyclic AMP binding to the amino-terminal domain induces an allosteric transition which changes the DNA binding property of the carboxy domains. We have isolated mutants in the crp gene called crp*, which make CRP functional in the absence of cyclic AMP. The mutations responsible for the CRP* phenotype cause substitutions by amino acids with bulkier side chains in the D -helix of the protein's carboxy domain, near the hinge which connects the carboxy to the amino domain. Apparently, the mutant CRP*s have assumed a conformation that is normally evoked by cAMP binding. Our study defines precisely the amino acids and thus the α-helicles which interact specifically to cause the allosteric shift.

We have found that a NusA amber fragment is still functional for anti-termination when expressed from a multicopy plasmid. This suggests that it is the aminoterminal portion of NusA that is important for anti-termination.

We have shown by pulse-labeling of RNA and by DNA-RNA hybridization, as well as by operon fusion analysis, that the autogenous regulation of rho is at the level of transcription. We have also found, by similar analysis, that cyclic AMP is a positive effector for rho gene expression, but also acts as a "repressor" of rho mRNA translation. This opposite control of the rho gene by cyclic AMP would maintain a constant level of Rho in cell. We are currently studying the system in vitro.

PHS 6040 (Rev. 1/84) GPO 914-916

Project Description

Objectives:

Gene expression in E. coli and bacteriophage λ is modulated at the level of transcription initiation, transcription termination and translation. Cyclic AMP, CRP, Rho and Nus gene products of E. coli, and N gene product of phage λ control one or more of these regulatory processes. Nus, Rho and N are involved in transcription termination and anti-termination. cAMP and CRP either stimulate or inhibit transcripition initiation. Frequently, the level of these regulatory proteins are critical in gene control. In order to understand the precise molecular basis of these and other regulatory biochemical reactions, we are studying (a) the structure of these genes, (b) how these genes are themselves regulated to vary their products, (c) their biochemical activity, and finally, (d) how these activities are biochemically regulated.

Methods Employed:

Microbial genetic and biochemical techniques. Also employs both in vivo and in vitro recombinant DNA technology, including dideoxy DNA sequencing method of Sanger.

Major Findings:

(1) It is known that CRP undergoes a conformational change when its effector molecule, cAMP, is added. In the cAMP-induced conformation, CRP binds to specific sites at the promoters of operons, and causes activation or inhibition of transcription. We have isolated mutations in the crp gene which allow CRP to function in the absence of cAMP. These mutations, which we call crp*, have been characterized. The crp* mutations were crossed from the bacteriophage vector in which they were isolated into the chromosome of an E. coli strain, which had the cya gene encoding adenylate cyclase deleted, for study of the CRP* phenotype. All crp* mutants could utilize the sugars lactose, sorbitol, and arabinose, but not maltose. Exogenously added cAMP enhanced the sugar utilization, and permitted maltose metabolism. Exogenously added cGMP also significantly enhanced sugar utilization, suggesting that the mutant CRP*s, unlike CRP^+, could use cGMP as an effector molecule.

The crp* genes and surrounding regions of four of the mutants were sequenced. All four had changes solely within the crp structural gene. Two of the mutants had single base changes, resulting in changes at amino acids 141 and 144 of CRP. The other two mutants had non-tandem double base changes. In one, amino acids 142 and 144 were altered; in the other, amino acids 141 and 72 were altered. These changes show:

(a) Substitution of an amino acid with a bulkier side chain at position 144, 141, or 142 and 144 is sufficient to allow CRP* to function without cAMP. These amino acids are located at the amino end of the D α-helix of the carboxy domain of CRP near the hinge region which connects the amino and carboxy domains of the protein. This area is not involved in cAMP binding

or DNA binding. We believe that the mutants which have changes in amino acids 141 or 142, because of the bulkier side chains of the substituted amino acids, have either an increased distance or an altered orientation between the D α-helix and the C α-helix of the amino domain. Similarly, the mutants with a change in amino acid 144, because of the bulkier side chain of the substituted amino acid, have an increased distance or orientation between the D α-helix and the F α-helix of the carboxy domain. We think that the CRP*s have, at least in part, assumed the conformational change that is caused by cAMP, and we have a clue as to how cAMP actually effects the conformational change in the protein.

(b) Mutation at position 72 does not seem to inhibit cAMP binding to CRP*. This result is somewhat surprising because amino acid 72 is in the cAMP binding site of CRP (as shown crystallographically by Steitz and coworkers), and the same amino acid (glutamic acid) is conserved in this position in the regulatory subunits of the cAMP-dependent protein kinase of animal cells. This may cause some re-thinking as to how cAMP is actually bound within CRP.

Starting with the crp* mutants, we have isolated secondary mutations within the crp gene, of two types:

(i) Stronger crp* mutants. These were isolated as those able to utilize the sugar maltose, in the absence of exogenous cyclic nucleotide.

(ii) Pseudorevertants. These were isolated as those which have regained the wild type phenotype, i.e., those which need cAMP to fuction. We can still rescue the crp* mutation from these, so we know it is there, but it is being suppressed.

Analysis of both of these types of mutants will enable us to prove our model of how CRP* works independently of cAMP.

(2) We believe that since the crp* mutants can be enhanced for utilization of sugars such as lacotse, arabinose, maltose, and sorbitol by exogenous cGMP, they might be using the low levels of endogenous cGMP when no exogenous cyclic nucleotide is present. Based on this assumption, we have isolated mutants, starting from a crp*-containing strain, which cannot use these sugars unless cGMP is added. These mutants could potentially be in a gene encoding guanylate cyclase or something regulating guanylate cyclase, since this is the phenotype of a cell which lacks cGMP. There is guanylate cyclase and cGMP in E. coli, but the function of cGMP in bacteria has remained obscure, since there have been no mutants in cGMP identified. These potential cGMP mutants which we have isolated are currently being studied to see whether they are indeed lacking cGMP.

(3) We had previously shown that strains which have mutations in the cya or crp genes make more Rho protein than do wild type cells, but there is less rho mRNA in these strains. Using both pulse-labeling of rho mRNA and operon fusion methods, we have shown that there is actually less rho mRNA transcribed in these strains. This suggests that CRP·cAMP may be acting to positively regulate rho transcription, but then is negatively regulating rho

at some post-transcriptional step. To determine if this negative effect is on the translation of rho, in vitro experiments using cell-free extracts from rho and crp mutants, as well as from the wild type strains, are being performed. We have prepared active S30 from wild type, crp$^-$ and rho$^-$ strains, monitoring β-lactamase synthesis. Preliminary cell-free studies of rho gene expression using a rho DNA template are in agreeement with the in vivo findings.

Significance to Cancer Research and the Program of the Institute:

National Cancer Plan Objective 3, Approach 1.

In cancer cells the expression of some genes are permanently turned on, i.e., expressed contitutively and some genes are permanently turned off, i.e., never expressed. Our studies are aimed at understanding the molecular basis of how genes are turned on and off and how genetic regulatory elements interact with each other. This understanding might help to prevent conversion of normal cells to those capable of forming cancers.

Proposed Course of Research:

(1) To determine the complete structure of the E. coli, rho, cya, crp and nus genes.

(2) To determine the regulation of the expression of these genes.

(3) To understand the interaction between these gene products for their biochemical activity.

Publications:

Garges S. and Adhya, S.: The regulation of crp and cya gene of Escherichia coli. Proceedings of the XV International Congress of Genetics. New Delhi, India, Oxford and IBH Publishing Company, 1984, pp. 19-33.

Garges, S. and Adhya, S.: Sites of allosteric shift in the structure of the cyclic AMP receptor protein. Cell. In press.

Gulletta, E., Gabriella, S., and Adhya, S.: Cloning and expression of the Escherichia coli rho gene in a plasmid vector. Microbiologia. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08751-05 LMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of the gal Operon of Escherichia Coli

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Adhya	Chief, Developmental Genetics Section	LMB, NCI
OTHER:	A. Majumdar	Visiting Scientist	LMB, NCI
	M. Polymeropoulos	Visiting Fellow	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Developmental Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are studying the control mechanisms of the expression of the gal operon of E. coli. We have demonstrated that the operon is controlled by two promoters, which are modulated by cyclic AMP in opposite ways. Both the promoters are negatively regulated by a gal repressor protein. We have also previously shown that each of the two gal promoters is negatively regulated by two operator elements; one of which (O_E) is located upstream to the promoters and the other (O_I) inside the galE structural gene. The existence of the two operators, which do not overlap with the promoters, have now been shown by three ways: (i) Genetic and DNA sequence studies of operator mutations; (ii) Repressor-operator interactions with purified repressor protein and operator DNA fragments; and (iii) Chemical protection of the operator DNA segments by repressor from DNase digestion and phosphate ethylation. These results also show that repressor binds to one and the same side of the two operators. The repressor does not compete with cAMP-CRP or RNA polymerase to bind to gal DNA. O_E and O_I are separated by 96 bp. Insertion of a 15 bp fragment, which introduces an extra half of a DNA double helical turn between them causes derepression of the operon. Taken together, our results suggest that gal repressor does not sterically hinder the binding of the other proteins to ensue repression. We have proposed that repressor molecules bound to O_E and O_I interact with each other to generate a DNA loop, which includes the promoters. This changes the conformation of the DNA structure, thus making it inadequate for promoter activity.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

We believe that the expression of most genes in E. coli is regulated in one way or another. Qualitatively different mechanisms have been discovered, e.g., positive and negative control at the level of both initiation and termination of gene transcription. Control mechanisms also exist at the level of mRNA processing and translation. We have demonstrated many of these control mechanisms using the galactose (gal) operon of E. coli. Using this model system, we are studying the molecular basis of the following regulatory mechanisms: (a) How cyclic AMP and CRP catalyze/inhibit transcription initiation. (b) How Rho protein of E. coli modulates transcription termination. (c) How gal repressor inhibits transcription initiation. (d) The nature of protein-protein and protein-nucleic acid interactions that bring about the above control mechanisms.

Methods Employed:

Microbiological, genetic, and biochemical techniques. Also employs both in vivo and in vitro recombinant DNA technology and DNA sequencing methods of Sanger.

Major Findings:

(1) We have discovered a new control of the gal operon. The expression of the gal operon is derepressed in mutant cells deficient in both cyclic AMP and the transcription termination factor Rho. We have proposed that the cyclic AMP repressible gal promoter, P_{G2}, may also be under Rho dependent transcription attenuation control. We are currently identifying the termini of the gal transcripts and studying the role of Rho in controlling P_{G2} in cell free systems.

(2) From genetic studies, we have previously postulated the existence of two active operator elements (O_E and O_I) in gal. Now we have confirmed that by binding of purified gal repressor to the operators:

(a) PAGE Analysis. Binding of a sequence-specific protein to DNA characteristically reduces the electrophoretic mobility of DNA fragments on a polyacrylamide gel. We have shown that gal repressor reduces the mobility of DNA fragments carrying the wild-type sequence of O_E, O_I, or both, in characteristic fashions. There was no characteristic mobility shift if the DNA fragments carried O^C mutations. These results demonstrate sequence-specific binding of repressor to O_E and O_I. These results have now been published.

(b) Ethylation and DNase Protection. The specific contacts of gal repressor to the two operators have been studied by protection of the corresponding phosphates in the DNA backbone by repressor from ethylation by ethyl-nitroso-urea. The operator segments protected from DNase I digestion by repressor have also been detected. These results show that repressors, in each case, occupy one and the same face of the helix.

(c) We have previously shown that repressor does not inhibit gal expression by inhibiting the binding of cAMP-CRP or RNA polymerase to the promoters. We have now shown that when an extra half DNA turn is induced between the two operator loci, which are normally separated by 93 bp, it causes derepression of the gal genes. These results are consistent with a model that gal repressor acts by affecting the promoter DNA structure from distal sites. Repressor bound to O_E and O_I may interact by protein-protein contact to generate a DNA loop structure of the promoter, making it inadequate for promoter activity. We are currently attempting to demonstrate such a structure by protein cross-linking and by electron microscopic studies.

(3) During the course of our studies on gal regulatory mechanisms, we have developed a two-step promoter cloning vector system. First, a promoter is cloned into a plasmid at a unique restriction site, which is flanked by gal and lac operon structural genes oriented in opposite directions. The introduction of a promoter between the two divergent lac and gal structural genes turns on either or both of the latter depending on the structure of the promoter DNA cloned. The Lac^+ and/or Gal^+ phenotype of the plasmid-carrying cells are easily detected on MacConkey Galactose indicator plates containing Indolyl-galactoside. A λ vector has been constructed into which the promoter fused to the lac/gal genes can be incorporated by in vivo recombination in a nonselective way using the amp^R as a selective marker. A lysogen of the resulting phage is studied for regulation of the promoter by quantifying the gal and/or lac enzymes. Although we have routinely used this vector system to study wild-type and mutant gal regulatory regions, the system can be used for any promoter in multicopy plasmid or single copy chromosomal system.

(4) We have determined the complete DNA sequence of the entire gal operon, which consists of 4000 base pairs.

Significance to Cancer Reseach and the Program of the Institute:

National Cancer Plan Objective 3, Approach 1. In cancer cells, the expression of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using the gal operon as a model system. This understanding might help to prevent the conversion of normal cells to those capable of forming cancers.

Proposed Course:

(1) To deterimine the complete structure of the gal operon. (2) To identify all the regulatory molecules involved in turning on and off the genes of the gal operon. (3) To understand the biochemistry of each of the regulatory steps. (4) To study the protein-DNA interactions between the repressor and the operator.

Publications:

Majumdar, A., and Adhya, S.: Demonstration of two operator elements in gal: in vitro repressor binding studies. Proc. Natl. Acad. Sci. USA 81: 6100-6104, 1984.

Adhya, S., Majumdar, A., Polymeropoulos, M., Orosz, L., and Itani, M: Mechanisms of gal Repressor Action. ICN-UCLA Sumposium. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08752-05 LMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanism of the Transport of Thyroid Hormones into Animal Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Sheue-yann Cheng Research Chemist LMB, NCI

COOPERATING UNITS *(if any)*
Laboratory of Cell Biology, DCBD, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.2	4.2	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unraducad type. Do not exceed the space provided.)*

We have demonstrated previously the presence of plasma membrane receptors for 3,3'5-triiodo-L-thyronine (T_3) in cultured rat GH_3 and human epithelioid carcinoma A431 cells. To further characterize the plasma membrane T_3 receptors in GH_3 cells, binding of [^{125}I]T_3 to the CHAPS solubilized receptors was evaluated. One class of binding sites was detected with a Kd of 7nM and a Bmax of 0.84 pmoles/50 ug protein. Analogs specificity, salt, pH and temperature dependency of binding were also examined.

The intracellular routing of T_3 in GH_3 cell during internalization was examined by using centrifugation in colloidal silica gel gradients. At 4°C, T_3 is associated with a membrane fraction enriched in adenylate cyclase activity indicative of plasma membranes. At 37°C, T_3 is internalized and associated with vesicular structures enriched with galactosyl transferase activity. Concomitantly, T_3 is incorporated into nuclei in a time-dependent manner. Thus, the association of T_3 with the nuclear fraction probably occurs by a receptor-mediated transfer of the hormone through vesicular structures rather than by a diffusion process.

Plasma membrane T_3 receptors in A431 cells were purified to homogenity by gel filtration, ion exchange chromatography and preparative electrophoresis. The partial amino acid sequence was determined by micro sequencing technique. Polyclonal and monoclonal antibodies against the purified receptors were raised. Using these reagents, the function and regulation of plasma membrane T_3 receptors are being studied.

Other Professional Personnel:

I. Pastan	Chief, LMB	LMB, NCI
M. Willingham	Chief, UC Section	LMB, NCI
R. Alderson	Guest Researcher	LMB, NCI
S. Hasamura	Visiting Fellow	LMB, NCI
S. Kitagawa	Visiting Fellow	LMB, NCI
E. Appella		LCB, DCBD, NCI

Project Description

Objectives:

To elucidate the mechanism of the entry of thyroid hormones into cells at molecular level. To study the mechanism of the translocation of thyroid hormones from cytoplasm into nucleus where the initiation of biological actions occur. To understand the mechanism of thyroid hormone action on cell growth.

Methods Employed:

Use radiolabled T_3 to quantify and characterize the binding of thyroid hormone to the detergent solubilized receptors. Synthesize affinity labeling reagents to label the receptors as marker for large scale purification. Use immunoprecipitation and metabolic labeling to screen and identify antibodies against receptors produced in rabbits and mice.

Colloidal silica gradients were used to separate the subcellular fractions where thyroid hormone and its receptors are associated. Fluorescence and electron microscopy were used to identify and localize the distribution of T_3 receptors in cells.

Major Findings:

Using [^{125}I]T_3 the binding of thyroid hormone to the CHAPS solubilzed plasma T_3 receptors was shown to be saturable and stereospecific. T_3 binds to the receptor with a Kd of 7nM and Bmax of 0.84 pmoles/50 µg protein. T_4 and D-T_3 are only 1/5 and 1/100, respectively, as effective as T_3 in binding to the solubilized receptors. The pH optimun for binding is 7.6. The binding activity is head sensitive and is lost upon pronase treatment indicating that the binding component is a protein. These binding characteristics are similar to those of thyroid hormone nuclear receptors.

At 37°C, translocation of T_3 from plasma membrane to nuclei in GH_3 cells is time-dependent. After 5 to 10 min at 37°C, T_3 started to accumulate in a nuclear fraction reaching a plateau after 40 min. The nuclear fraction has 11-12% of total cellular T_3. The transfer of T_3 from plasma membrane to nuclear fraction probably is via golgi-lysosome system. Thus the association of T_3 with the nuclear fraction probably occurs by a receptor-mediated transfer of the hormone through vesicular structures rather than by a diffusion process.

The purified plasma membrane T_3 receptors from A431 cells have an apparent molecular weight of 55,000 as determined by SDS-PAGE and Sephadex G-200 chromatography. The homogenity is shown by one N-terminal sequence and one commassie-stained band in 2-D gel. Four to six hybridoma clones secreting antibodies against the purified receptors were obtained in 3 fusions. A high titered-polyclonal antibody against the purified receptors was also obtained.

Significance:

National Cancer Plan Objective 5, Approach 5.

Transformation of cultured cells appears closely linked with the ability to form malignant tumors in vivo. The understanding of the basic mechanisms in hormone-receptor interactions which result in controlled or uncontrolled cell growth is likely to be of great value in understanding the basic mechanisms leading to the formation of cancer cells. The knowledge gained from such studies is likley to have great impact on the ability to design or modify therapeutic procedures.

Proposed Course:

We will continue to study the molecular mechanism of the transport of thyroid hormones into animal cells. The polyclonal and monoclonal antibodies will be used to study the intracellular distribution of T_3 receptors in cultured cells and various animal tissues. Oligonucleotides with the sequences corresponding to the N-terminal and other sequences of the plasma membrane T_3 receptors will be synthesized and used as probes to clone the genes of T_3 receptors. This should lead to the understanding of structure, function and regulation of the plasma membrane thyroid hormone receptors.

Publications:

Cheng, S.-y and Lai, C.S.: Molecular dynamics of 3,3',5-triiodo-L-thyronine in model membranes: A spin label study. Arch. Biochem. Biophys. 232: 477-481, 1984.

Hasumura, S., Rossi, B., Alderson, R., Pastan, I., and Cheng, S.-y: Antibodies against the plasma membrane 3,3',5-triiodo-L-thyronine binding protein of rat pituitary GH_3 cells: partial characterization and cross-species immunoractivity. Biochem. Biophys. Res. Commun. 124: 956, 1984.

Cheng, S.-y and Alderson, R.: Molecular characterization of plasma membrane 3.3'5-triiodo-L-thyronine binding sites in cultured cells. J. of Endocrinological Investigation 7: 21, 1984.

Cheng, S.-y: Structural similarities between the plasma membrane binding sites for L-thyroxine and 3,3'5-triiodo-L-thyronine in cultured cells. J. Rec. Res. 5: 1, 1985.

Cheng, S.-y., Willingham, M.C., Lai, C.S., and Pastan, I.: Receptor-mediated uptake of 3,3'5-triiodo-L-thyronine by cultured fibroblasts. In Miyamoto, H. (Ed.): Proceedings of the Ninth Taniguchi Symposium. Japan, Res. Dev. Corp., 1985, p. 58.

Alderson, R., Pastan, I., and Cheng, S.-y: Characterization of 3,3'5-triiodo-L-thyronine binding site on plasma membranes from human placenta. Endocrinology. 116: 2621, 1985

Stevens, R.L., Hasamura, S., Parsons, W.G., and Cheng, S.-y.: The Identification of a Plasma Membrane 3,3'5-Triiodo-L-Thyronine Receptor on the Cultured Swarm Rat Chondrosarcoma Chondrocyte and the Lack of its Up Regulation by Insulin In Vitro*. Endocrinology. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08753-03 LMB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chrecters or less. Title must fit on one line between the borders.)*
Immunotoxin Therapy of Cancer Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ira Pastan	Chief, Laboratory of Molecular Biology,	NCI
OTHER:	M. Willingham	Chief, UCS,	LMB, NCI

COOPERATING UNITS *(if any)*
Columbia Med. School
U.S. Army Med. Res. Inst. of Infect. Dis., Ft. Detrick, MD
Med. Branch, DT/NCI
Metabolism Branch, DCBD/NCI
Lab. Theoretical Biol. DCBD/NCI/NIH
Cetus Corp.

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.1	4.1	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Pseudomonas toxin has been coupled to monoclonal antibodies to make immunotoxins. When coupled to an antibody to the T cell growth factor receptor to make anti-TAC-PE, it kills leukemia cells that are TAC positive. When injected into monkeys, the immunotoxin has very little toxicity and good survival indicating it may be a useful agent for treatment of this type of leukemia.

Adenovirus enters cells in the same vesicles (receptosomes) as these immunotoxins. By lysing receptosomes, adenovirus efficiently releases the immunotoxin into the cytosol and selectively increases cell killing. The penton base of adenovirus is important for vesicle lysis. Adenovirus at pH 5 will permeabilize the plasma membrane of KB cells to small molecules.

PHS 6040 (Rev. 1/84)

GPO 914-918

Other Professional Personnel:

D. FitzGerald	Staff Fellow	LMB, NCI
P. Seth	Visiting Fellow	LMB, NCI
S. Akiyama	Visiting Fellow	LMB, NCI
R. Pirker	Visiting Fellow	LMB, NCI
S. Leppla	U.S. Army Medical Research Institute of Infective Diseases, Fort Detrick, MD	
T. Waldmann	Metabolism Branch	DCBD, NCI
R. Ozols	Medicine Branch	NCI
T. Hamilton	Medicine Branch	NCI
W. Laird	Cetus Corporation, Palo Alto, CA	
A. Frankel	Cetus Corporation, Palo Alto, CA	
R. Blumenthal	Lab. Theoretical Biol.	DCBD, NCI

Project Description

Objectives:

To develop methods of selectively killing human cancer cells, and to determine the biochemical basis of this effect.

Methods Employed:

Preparation of toxins, antibodies and toxin-antibody conjugates; growth and purification of adenovirus; chemical modification of toxins and virus, protein purification and cell culture, testing of immunotoxins in nude mice bearing human tumors.

Major Findings:

Having previously demonstrated that an immunotoxin composed of Pseudomonas toxin (PE) and an antibody to the interleukin 2 receptor (anti-Tac) kills leukemia cells displaying the Tac receptor we began toxicity studies in monkeys to evaluate the usefulness of this reagent in patients. Four monkeys weighing 3-4 kilos were injected intravenously with 100 µg to 500 µg PE-anti-Tac. All tolerated the agent well. There was a transient increase in white blood cells with a fall in lymphocytes and an increase in granulocytes. Temperature rose 1-2°C for 1-2 days. There was also a transient rise in serum glutaminase levels. Autopsies were performed at 5-6 weeks. In animals given a single dose no pathological changes were noted. In one animal given four (4) 100 µg doses some changes in arterioles were noted consistent with a mild immune response to the reagents injected. In the monkey given 500 µg PE-anti-Tac, the plasma levels of PE-anti-Tac were measured over a 4 day period. The drug fell from 2-3 µg/ml at one hr to 200 ng/ml at 24 hrs. These studies indicate PE-anti-Tac might be a safe and effective agent to treat adult T-cell leukemia.

In collaboration with Cetus Corporation, we have evaluated the activity of a group of monoclonal antibodies conjugated to ricin A chain or to Pseudomonas toxin on ovarian cancer cells. These studies show that PE conjugates are

3- to 10-fold more active than ricin A conjugates. Animal experiments are underway to evaluate the activity of some of these agents.

To determine how adenovirus (Ad_2) enters cells and lyses endocytic vesicles (receptosomes) which have an acidic pH, we have devised a new and simple assay. We find Ad_2 directly permeabilizes the plasma membrane of cells when cells are placed at pH 5-6 but not at pH 7.4. Small molecules but not large molecules are released from the cells indicating the "holes" created are small. Why adenovirus lyses endocytic vesicles but only makes "small" holes in the cell membrane is not yet clear and merits further study. To determine the biochemical nature of the interaction of Ad_2 with membranes we measured its interaction with a detergent, Triton X114. We find that the virus binds to this detergent at pH 5 but not at pH 7. When the the viral coat proteins were separated, each was found to have this property with penton base protein showing the strongest binding at pH 5. These studies suggest that Ad_2 penetrates the lipid bilayer as the pH in the endocytic vesicle in which it is internalized falls. In collaborative studies with R. Blumenthal, we have examined the action of adenovirus on liposomes and have found that the virus will rapidly lyse liposomes at pH 5-6 but not at pH 7. These studies help support the hypothesis that the low pH of the receptosome is required for adenovirus to penetrate the membrane and cause vesicle lysis.

Proposed Course:

To test PE-anti-TAC in patients with adult T cell leukemia. To test immunotoxins in animals that are active against ovarian cancer cells in tissue culture. To identify agents that increase the activity of immunotoxins.

Publications:

Seth, P., FitzGerald, D., Willingham, M.C., and Pastan, I.: Pathway of adenovirus entry into cells in Concepts in Viral Pathogenesis, Vol II. In press.

Pirker, R., FitzGerald, D.J.P., Hamilton, T.C., Ozols, R.F., Bjorn, M.J., Frankel, A.E., Willingham, M.C., and Pastan, I.: Characterization of immunotoxins active against ovarian cancer cell lines. J. of Clinical Investigation. In press.

Seth, P., Pastan, I., and Willingham, M.C.: Adenovirus-dependent increase in the cell membrane permeability. J. Biol. Chem. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08754-02 LMB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic Analysis of the Multiple Drug Resistance Phenotype in Tumor Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M. M. Gottesman — Chief, Molecular Cell Genetics Section — LMB, NCI

I. Pastan — Chief, Laboratory of Molecular Biology — NCI

COOPERATING UNITS *(if any)*

Division of Cancer Therapy, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	6.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

During the course of chemotherapy of human cancers, variants which are resistant to multiple drugs frequently arise. We have been investigating the genetic and biochemical basis for this pleiotropic resistance of human tumor cells to chemotherapeutic agents. A model system using the cultured KB cell, a human carcinoma cell line, has been developed in which mutant cells selected independently for resistance to high levels of either colchicine, adriamycin or vinblastine have also been found to be cross-resistant to colchicine, adriamycin, vincristine, vinblastine, puromycin and actinomycin-D. Genetic linkage of these multiple drug resistances in the colchicine-selected line has been demonstrated by their coordinate appearance, coordinate reversion, codominance and cosegregation in somatic cell hybrids, and cotransfer by DNA-mediated gene transfer. Accumulation of several of the drugs involved in the multiple drug-resistance phenotype has been shown to be reduced in the KB cells. Drug-resistance correlates with the loss of a family of glycoproteins of molecular weights 70-80,000 on the cell surface detected by polyclonal and monoclonal antibodies and, in some cases, the appearance of a low molecular weight protein (p21). Drug-resistance also correlates with amplification of a DNA segment detected either by an *in gel* renaturation technique or on Southern blots using probes cloned from amplified DNA in drug-resistant hamster cells.

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GPO 914-918

Other Professional Personnel:

N. Richert	Senior Investigator	LMB, NCI
T. Fojo	Medical Officer	LMB, NCI
D.-W. Shen	Fogarty Fellow	LMB, NCI
C. Cardarelli	Research Biologist	LMB, NCI
J. Hwang	Guest Researcher	LMB, NCI
D. Atlas	Guest Researcher	LMB, NCI
M. Cornwell	Guest Researcher	LMB, NCI

Project Description

Objectives:

To use genetic and biochemical analysis of human drug-resistant cell lines and tumor tissues to understand the molecular basis of resistance to chemotherapy in tumors.

Methods Employed:

Somatic cell genetic analyses including tissue culture, mutant isolation, karyotyping, cell hybridization and gene transfer, standard biochemical techniques including gel electrophoresis and chromatography, and recombinant DNA technology including cDNA and genomic cloning, Southern and Northern blots, hybrid selection and translation and DNA sequencing.

Results:

(1) Through sequential, single-step selections, we have developed human KB carcinoma cell lines which are several hundred-fold more resistant to either colchicine, adriamycin or vinblastine than the parent line. These cells are all highly cross-resistant to colchicine, vincristine, vinblastine, adriamycin, puromycin and actinomycin D. These resistances are genetically linked as shown by coordinate appearance, coordinate disappearance in the absence of the selective agent, codominance and cosegregation in somatic cell hybrids, and cotransfer by DNA mediated gene transfer. The early steps of multiple drug resistance in KB cells occur at low frequency and require mutagenesis with EMS; later steps occur easily and can be facilitated by use of the tumor promoter mezerein, which is known to facilitate the amplification of genes in mammalian cells. These data taken together suggest that early steps in development of multidrug resistance may involve mutation and later steps could involve gene amplification.

(2) Several lines of evidence have been accumulated to support the two-step model of development of multidrug resistance (mutation followed by amplification). Evidence for gene amplification includes the appearance of double minute chromosomes in our high-level multidrug resistant cell lines, as well as evidence of gene amplification obtained by an *in gel* DNA renaturation technique which detects DNA segments amplified at least 30-50-fold in

colchicine, vinblastine and adriamycin-selected multidrug resistant lines. Using genomic probes originally isolated by Roninson (Nature, 309, 626, 1984) from multidrug resistant hamster cells, or obtained from the in gel renaturation analysis of our human

KB cell lines, it is possible to demonstrate high-level gene amplification (>50-fold) on Southern blots of highly resistant colchicine, vinblastine or adriamycin-selected KB cell lines. At low levels of resistance gene amplification is not seen, but increased expression of a 4.5 kb mRNA deteced by the same genomic probe is observed. These data suggest that in the KB system low-level resistance arises by increased expression of a cellular gene, whereas high-level resistance requires its amplification.

(3) The sequential development of multiple drug-resistance is correlated with decreased accumulation of drugs due to decreased uptake and increased efflux by the resistant cells. This accumulation defect is reversed by treatment of cells with the Ca^{2+}-antagonist verapamil, which also reverses the drug-resistant phenotype of the cells. These data suggest that drug resistance is due to a membrane alteration or alterations which affect drug uptake and efflux in the KB cells.

(4) High resolution two-dimensional gel analysis of drug-resistant cell lines suggests that a small number of specific protein alterations are associated with the development of multiple drug resistance. These include the loss of a family of cell surface glycoproteins of molecular weights 70-80,000 and the appearance of a protein of molecular weight 21,000 in some cell lines. Polyclonal and monoclonal antibodies have been developed to the 70-80,000 dalton glycoprotein.

(5) We have prepared complete cosmid libraries from multidrug-resistant cell lines and are screening these for the presence of amplified genes and for genes which confer drug resistance in gene transfer experiments.

Significance to Cancer Research and the Program of the Institute:
National Cancer Plan Objective 3, Approach 3.5.

The goal of these studies is to understand the basis of multiple drug-resistance in chemotherapy-resistant human tumors so that rational therapy can be devised to prevent or circumvent the multiple drug-resistance phenotype. Dramatic success in treatment of some human tumors with chemotherapy suggests that chemotherapy can be curative in many more cases if steps can be taken to reduce the appearance of drug resistant tumor cells. One possible approach, if a cell surface protein is responsible for multiple drug-resistance, would be to use toxin-antibody complexes to kill cells with the antigen in question on their surfaces. Another approach would be to use cloned DNA probes for drug-resistance genes to detect their expression before treatment and modify treatment modalities accordingly.

Proposed Course:

To continue to isolate human uptake and efflux and to develop an in vitro system to study drug accumulation in membrane vesicles derived from multidrug-resistant cells; to isolate human genes coding for multiple drug-resistance by gene transfer and rescue techniques and by screening cosmid libraries for genes known to be amplified in our drug-resistant lines; to determine the protein product of the amplified genes in multidrug-resistant lines.

Publications:

Akiyama, S.-I., Fojo, A., Hanover, J.A., Pastan, I. and Gottesman, M.M.: Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. Somat. Cell and Molec. Genet. 11: 117-126, 1985.

Richert, N., Akiyama, S.-I., Shen, D.-w., Gottesman, M.M., and Pastan, I.: Multiply drug-resistant human KB carcinoma cells have decreased amounts of a 75-kDa and a 72-kDa glycoprotein. Proc. Natl. Acad. Sci. USA 82: 2330-2333, 1985.

Fojo, A., Akiyama, S.-i., Gottesman, M.M., and Pastan, I.: Reduced drug accumulation of multiply drug-resistant human KB carcinoma cell lines. Cancer Research. In press.

SUMMARY

Annual Report of the Laboratory of Biochemistry, National Cancer Institute
October 1, 1984 to September 30, 1985

I. INTRODUCTION

In this Laboratory the tools of biochemistry, genetics and molecular biology are used to study fundamental questions in biology using a variety of organisms. The areas being investigated include development and differentiation, DNA replication, the biosynthesis of macromolecules, the regulation of cellular processes at the level of gene expression and macromolecular interactions, the organization of eukaryotic genomes and the biology of T-cells. In addition, there is an interest in the development of methods, particularly those for protein separation. In spite of this large and diverse scientific activity, the atmosphere fosters exchange, collaboration and collegiality through common methodological approaches, a full schedule of journal clubs, seminars, group meetings and presentations of work-in-progress, and by a long tradition of common use of equipment and supplies. The advantages of such diversity have become especially apparent during this last year. In several research areas, it became apparent to the investigators that a shift in approach would be productive. Thus, we have enzymologists and protein chemists now using the techniques of molecular genetics while several of the traditional molecular biologists are beginning to purify proteins. There is no doubt but that these changes are markedly facilitated by the presence of knowledgeable colleagues.

The Laboratory is divided into seven sections. In one of these, the Section Chief is the only independent investigator. In the others, several investigators direct independent and distinct research programs. Each independent investigator initiates and carries out his or her own program, usually with the assistance of one skilled technician and the collaboration of several post-doctoral fellows or students. The students are enrolled in the FAES-Johns Hopkins Joint Program. Each group typically has between 3 and 8 people altogether.

Dr. Michael Yarmolinsky joined the Laboratory in late 1984, as an independent investigator and Chief of the Section on Developmental Biochemistry and Genetics. This appointment fills the vacancy formed by the departure of Igor Dawid several years ago. Along with Dr. Yarmolinsky, Dr. Dhruba Chattoraj's arrival has renewed the vitality of this Section. We are still trying to recruit a senior staff member to replace Dr. Brad Thompson as Chief of the Section on Biochemistry of Gene Expression. At present this Section has no staff.

The major problems encountered during the last year are all related to the significant decrease in positions available for Staff Fellows. As a result of this situation, our training efforts are largely directed toward foreign post-doctoral fellows. We have succeeded in attracting some young Americans who have fellowships from independent sources. Nevertheless, the consistent staffing of the laboratories at an appropriate level remains problematic. The situation is exacerbated by the unprogrammed and intermittent imposition of hiring freezes which destroys efforts to plan rationally and recruit high quality staff.

II. BIOSYNTHESIS SECTION (Dr. E.L. Kuff, Chief)

The Section has four permanent senior investigators and is divided into three independent research groups.

A. Dr. Beverly Peterkofsky and coworkers: Two different aspects of regulation of collagen synthesis are being studied. The objective of one project is to determine the mechanism by which vitamin C (ascorbic acid) controls connective tissue metabolism. Previously these workers showed that decreased collagen synthesis in parietal bone of scorbutic guinea pigs was directly related to the extent of weight loss during the third and fourth week of scurvy, rather than to defective proline hydroxylation. Current studies show that collagen synthesis in cartilage is similarly affected by scurvy and that synthesis of another major component of cartilage extracellular matrix, proteoglycan, is also decreased. Both effects are directly correlated with weight loss and synthesis of collagen and proteoglycans appears to be coordinately regulated. These, and other results, suggest that ascorbate deficiency indirectly produces these effects by inducing anorexia, which leads to a chronic fasting state. Acute fasting for 96 hr with ascorbate supplementation causes a similar coordinate reduction in collagen and proteoglycan production. Decreased collagen production in both bone and cartilage of acutely fasted animals is not due to an increase in degradation but to decreased synthesis caused by a reduction in the levels of procollagen mRNA.

In a second study, these workers found that in a nitroquinoline oxide transformant of BALB 3T3 (NQT-3T3), there is almost complete supression of synthesis of type I procollagen, the major product of the parent 3T3 cells. In addition, synthesis of two previously undescribed types of collagen is induced. Both of these molecules appear to have a procollagen type of structure. Each is composed of single subunits with a pepsin-resistant helical region having a typical repeating tripeptide sequence susceptible to bacterial collagenase, plus pepsin-sensitive noncollagenous regions. Further characterization of these novel collagen species is under way.

B. Dr. Samuel Wilson and coworkers: As part of a continuing study of the mechanism of mammalian DNA replication, Dr. Wilson and coworkers are currently interested in three relevant proteins, α-polymerase, β-polymerase and the single-strand nucleic acid binding protein termed helix destabilizing protein. Using appropriate monoclonal antibodies, they scanned a newborn rat brain cDNA library prepared in the expression vector λgt11. Six cDNAs for the binding protein were isolated and the nucleotide sequences of each was determined. One of the cDNAs selected for detailed study was a full-length copy of mRNA encoding the predominant species of the binding protein family in rodent tissue. Based on the nucleic aid sequence, this polypeptide has a molecular weight of 34,215; it is routinely purified as a truncated polypeptide of ~25,000-M_r.

A cDNA clone for rat β-polymerase (see previous Annual Report) was sequenced. This DNA appeared to be a full-length mRNA copy also, in this case encoding the 40KDa enzyme. Northern blot analysis of newborn rat brain polyA^+ RNA using this nick translated cDNA as probe revealed one predominant hybridizing RNA species of about the same dimension as the insert itself. Southern analysis of rat genomic DNA was consistent with a single copy gene. Similar results were obtained with

human genomic DNA. The gene was localized to human chromosome 8 using hybrid cell chromosome segregation techniques. β-Polymerase levels in a number of human cell lines were determined and were found to be modestly elevated (2 to 3-fold) in lung cancer cells and in cells from patients with ataxia telangectasis.

One cDNA clone for rat α-polymerase was also obtained. With this as probe, Northern blot analysis of newborn rat brain polyA$^+$ RNA revealed a single hybridizing species of mRNA of about 4600 bases. This mRNA was 4-times longer than the cDNA itself and was long enough to encode the 180 to 190KDa α-polymerase catalytic subunit described in last year's report. Southern blot analysis of rat genomic DNA was consistent with a single copy gene. In an attempt to obtain rat cell lines with amplified α-polymerase genes, these workers isolated cell lines resistant to the competitive inhibition of α-polymerase, butylphenyldeoxyguanosine. Two of these cell lines were found to have increases in the amounts of both α-polymerase enzyme activity and α-polymerase catalytic subunit as determined by Western blot analysis.

C. Dr. Edward L. Kuff and Dr. Kira K. Lueders and coworkers: Continued studies are in progress on Intracisternal A-particle (IAP) genes, a family of retrovirus-like genetic elements endogenous to mice and other rodent species. The past year saw a surprising development in which IAP genes were linked to a class of T-cell immunoregulatory factors that modify IgE production indifferentiating B-lymphocytes. Drs. Kevin Moore and Kimishiga Ishizaka (not NIH), who have been studying this regulatory pathway for some time, obtained from the mRNA of a rat mouse T-cell hybridoma a cDNA clone which coded for an IgE-potentiating factor. Comparison of the cDNA sequence with that obtained by Judy Mietz in Dr. Kuff's laboratory for an authentic endogenous mouse IAP gene established that the cDNA represented a modified IAP element and that the active polypeptide was a form of the IAP-related gag-pol fusion product. Antisera against purified IAP structural (gag) protein bound the IgE regulatory molecules produced not only by the cDNA clone when transfected into COS7 cells but by normal rat and mouse T-lymphocytes with stimulated appropriately in vivo. Dr. Kuff's group is seeking to determine whether analogous regulatory factors for other types of immunoglobulins are also IAP-related.

Dr. Kuff has also continued his collaboration with Dr. Edward Leiter of the Jackson Laboratory on the possible role of IAP antigen expression in the pathogenesis of a genetically determined insulin-dependent diabetes in mice. They have now shown that IAP mRNA and proteins are induced in isolated pancreatic islets incubated in a high-glucose medium and that both the basal and glucose-induced levels are significantly higher in islets from diabetes-susceptible mice than in those from resistant strains. They postulate that islet cells carrying glucose-induced IAP antigens on their cell surfaces may be subject to autoimmune attack.

Dr. Lueders has been studying a subset of mouse IAP genes characterized by deletions of various sizes and insertion of a particular 300 base pair sequence element which distinguishes this group, the so-called type II IAP genes, from the majority of IAP genes (type I) which lack this insertion. The inserted sequence element has not been detected outside of Mus musculus and some closely related mouse species. Type II genes show both deletions and rearrangements of IAP sequences; their complex structure suggests that they were formed through multiple recombinational events. Type I IAP genes contain at their 5' ends a 74 base pair region which contains a core enhancer sequence; this region is duplicated or triplicated in type II genes. A sub-class of type II IAP genes was associated with

other repeated elements: specifically 11 of 12 type IIC genes contained truncated L1 family members in their 5' flanking regions. Only 3 of 29 type I genes contained this repeat element. Only certain sub-classes of type II genes are transcribed, the lack of general type II gene activity may result from their association with L1 repeats which are highly methylated and poorly transcribed. An inhibitory effect of flanking DNA on provirus transcription has been noted by others.

III. CELLULAR REGULATION SECTION (Dr. O. Wesley McBride, Chief)

The work of the Cellular Regulation Section is carried out by two independent groups.

A. O. Wesley McBride and coworkers: A large group of independent somatic cell hybrid lines that segregate human chromosomes were isolated and the human chromosome content of each line was determined by isoenzyme analysis and karyotyping. Southern analysis of DNA isolated from these hybrids with molecularly cloned DNA probes was used to localize human genes to specific chromosomes with particular emphasis on genes potentially involved in human neoplasia. These include known protooncogenes, translocation breakpoints in human tumors, and genes involved in DNA replication or activation of carcinogens. Several genes have also been regionally localized on specific human chromosomes by in situ hybridization of the probes with human metaphase spreads and by analysis of hybrids containing specific chromosome breaks or translocations. These somatic cell hybrids have been used previously to chromosomally map eight protooncogenes, heavy and light chain immunoglobulin genes and pseudogenes, the α, β, and γ fibrogen genes, the calcitonin gene, and the metallothionein multigene family. The human gene for p53 tumor antigen, which is elevated in many transformed cells and tumors, now has been mapped to chromosome 17 and localized to band 17 p13 by both in situ hybridization and hybrid cell DNA analysis. The cytoplasmic P_1-450 structural gene has been assigned to chromosome 15, L-myc protooncogene to chromosome 1p32, and beta DNA polymerase gene to chromosome 8 (probably band 8q24). A sequence at the translocation breakpoint in follicular B-cell lymphomas has been mapped to chromosome 18 and it is covalently linked to the IgH sequences translocated from chromosome 14. The gene for human collagen type III has been localized to the long arm of chromosome 2 and a gene and pseudogene for J protein have been assigned to chromosomes 4 and 8, respectively.

The GL-13 BC guinea pig leukemia model has many similarities to human chronic myelogenous leukemia and efforts were made to confirm this relationship by molecular biological approaches. Southern analysis of guinea pig leukemic DNA with ^{32}P-labeled onc gene probes did not detect any rearrangements of c-abl or several other cellular protooncogenes. Efforts to detect alterations in the guinea pig c-abl transcript, similar to those consistently reported in human CML, are in progress. Three inserts representing different portions of v-abl have been cloned into riboprobe vectors preparatory to chromosomal mapping of c-abl in guinea pig leukemia cells.

B. Dean Hamer and coworkers: As a model for the regulation and function of eukaryotic genes, the metallothioneins (MT) are being analyzed by molecular genetic and biochemical approaches in both mammalian cells and yeast. Mutagenesis and gene transfer experiments have demonstrated that a duplicated upstream sequence is required for heavy metal induction of mouse MT-I gene transcription. Human MTs are encoded by a complex multigene family that includes at least five

functional members clustered on chromosome 16. Two highly homologous human MT-I isoform genes have been cloned and sequenced thereby permitting analysis of cell type-specific expression. Both genes are functional since they are transcribed in non-differentiated cells and following gene transfer. In contrast, a highly specific pattern of MT-I isoform expression was found in differentiated cell types. The specificity of expression correlated with differential metal-binding properties of these proteins. This was determined by comparing the stoichiometry of binding of different heavy metals to the MTs expressed in isogenic cell lines transformed with different MT isoform genes on bovine papilloma virus vectors. The structural basis of this phenomenon is being studied by constructing hybrid proteins carrying different domains from these isoform genes. Activated *ras* oncogenes in cells were also found specifically to enhance MT gene transcription and the molecular mechanism for this phenomenon is being investigated by treating normal cells with growth factors and other agents thought to be affected by *ras* expression. Studies are in progress to detect and isolate cellular factors involved in regulation of MT expression by heavy metals and other agents. Since the yeast *Saccharomyces cerevisiae* has significant genetic and technical advantages as a model system to study the function and regulation of a metallothionein-like protein, haploid and diploid yeast strains have been constructed in which this endogeneous gene (*CUP1*) has been deleted and replaced by a yeast selectable marker. The role of this protein (copperthionein) in heavy metal detoxification was confirmed by the high sensitivity of these mutant yeast strains to copper-poisoning whereas the absence of any essential physiological role of the protein under laboratory conditions was indicated by a normal growth and life cycle of the yeast mutants. To assess the possible role of copperthionein in regulating its own expression, a gene was constructed containing *CUP1* regulatory and promoter sequences fused to coding sequences of *galK*. Study of the expression of this gene introduced into yeast strains lacking or retaining the endogenous *CUP1* structural gene demonstrated that copperthionein protein negatively autoregulates its own expression. The *CUP1-galK* fusion gene was also used to study *cis*-acting control sequences responsible for regulation by copper. Analysis of an extensive series of deletion and linker scanner mutants revealed a complex set of both positive and negative regulatory signals. A *trans*-acting yeast mutant with an altered pattern of *CUP1* expression has been isolated and it has a phenotype similar to human cells from Menkes' disease (i.e. increased ^{64}Cu uptake, sensitivity to copper poisoning, and a high basal level of *CUP1* transcription). Classical genetic analysis indicates that a single co-dominant gene controls the phenotypic properties, and a gene that suppresses the phenotype has been cloned and is being sequenced. The yeast model has provided a means for studying the structural basis for the detoxification and autoregulatory functions of copperthionein. The protein has been purified to homogeneity and the amino acid sequence determined. Comparison with the sequence predicted from DNA sequencing indicates that the first eight amino acids are removed during processing. The native protein contains 8 atoms of copper per molecule. Analysis of the stoichiometry of binding of silver, cadmium and zinc to the apoproteins indicates that different heavy metals bind in distinct configurations. The structural requirements for metal-binding and the physiological importance of the processing step are under study using mutants involving the leader sequence and metal-binding sites. Studies also show that mammalian metallothionein can complement both known functions of the yeast gene thereby demonstrating functional analogy of the proteins and relevance of the comparative studies.

IV. DEVELOPMENTAL BIOCHEMISTRY AND GENETICS (Dr. Michael Yarmolinsky, Chief)

The Developmental Biochemistry and Genetics Section is made up of three independent research groups.

A. Dr. Michael Yarmolinsky and coworkers: Fertility factor F and prophage P1 are composite replicons that are similarly organized at a plasmid maintenance region, although essentially nonhomologous in their DNA sequences. Each can integratively suppress dnaAts mutations in E. coli. Particular isolated basic replicons from F and P1 that are similarly capable of dnaAts integrative suppression exhibit a single region of DNA homology that corresponds to a tandem pair of DnaA protein binding sites. This paradox prompted Dr. Yarmolinsky and Dr. Egon Hansen to reexamine the capacity of these and certain other replicons to function in dnaA null (insertion and deletion) mutants. They found that some replicons that are capable of dnaAts integrative suppression and that bear DnaA protein binding sites (R1, pBR322) can replicate in the total absence of dnaA function, whereas others cannot. Based on experiments with an E. coli dnaA that is integratively suppressed by a dnaA-independent mini-R1, they show that the ori-2 replicon of mini-F and the corresponding replicon of a mini-P1 fail to replicate when dnaA function is totally absent. They provide evidence that the requirement for DnaA protein in the replication of the mini-P1 is direct and not the result of an effect on the synthesis of RepA, the sole P1-determined protein required for the mini-P1 replication. Other replicons of F and P1 exhibit no absolute requirement for dnaA function. The different kinds of replicons that are present in F and P1 may serve to enlarge the range of bacterial hosts and environmental circumstances in which these plasmids replicate.

B. Dr. Carl Wu and coworkers: The sequential arrangement of nucleosomes along the chromatin fiber is punctuated by highly nuclease-sensitive sites. This group has previously mapped such sites to the 5' terminus of several heat shock genes in Drosophila by a novel indirect end-labeling technique. Such preferentially accessible sites in chromatin may function as points of entry to the DNA for RNA polymerase and control proteins. Members of the group have developed an exonuclease protection technique for mapping protein binding sites in chromatin, and have found two such sites for both the hsp 82 and hsp 70 genes. Site I is present before and after heat shock gene activation, and covers the TATA box sequence, whereas site II surrounds the upstream heat shock control element and appears only during heat shock. The group suggests that heat shock genes are activated by the sequential binding of at least two protein factors, and in the period covered has developed new chromatin-binding and DNA-binding procedures to detect these proteins in crude nuclear extracts. These procedures are now being used as assays for the purification of the two proteins using standard column chromatography and high performance liquid chromatography.

C. Dr. Bruce Paterson and coworkers: Using cloned cDNA probes this group has isolated the genomic sequences for the following proteins: alpha skeletal actin, alpha cardiac actin, beta cytoplasmic actin, myosin light chains 1-3, vimentin, pyruvate kinase, glyceraldehyde phosphate dehydrogenase, and a nerve growth factor induced gene. These genes have been partially or completely defined by DNA sequence analysis. The actin genes have been subcloned into the eukaryotic vectors PSV2gpt or PSV2neo and transfected into L-cells, into the C2 mouse muscle cell line, and into the C3H 10T1/2 mesodermal cell line. 5' and 3' probes specific for the various chicken actin genes have been used to measure levels of expression and to monitor the pattern of regulation of the actin genes in these

various mouse cell backgrounds. The entire sequence of the alpha cardiac actin gene was determined and compared to the alpha skeletal actin gene, the other muscle specific actin isoform. Even though both genes are expressed in developing muscle there is no sequence homology in the 5' or 3' flanking regions of the genes that suggests a common regulatory mechanism controlling tissue specific expression or developmental regulation. The pattern of expression for the transfected actin genes is dependent upon the cell background and this characteristic is being used to determine the regulatory regions in the various genes. The expression of the chicken beta actin gene decreases in parallel to expression of the mouse endogenous beta actin gene during myogenesis in all the mouse muscle cell lines tested. Promoter exchange studies have determined that this regulation is dependent upon elements 3' to the promoter itself and minigene constructions suggest the 3' noncoding region is important in this regulation. Nuclear run-on experiments demonstrate the polymerase loading of the gene decreases with myogenesis ruling out post transcriptional control of beta actin expression. The 3' end of the beta actin gene is under investigation. The promoters for the myosin light chains genes 1 and 3 are apparently not functional out of the context of the entire gene since in frame CAT constructs driven by each of the promoters are silent. This suggests promoter activity may be regulated by other sequences within the body of the LC1/LC3 gene. The myosin heavy chain II gene of acanthamoeba, a nonmuscle myosin gene, has been isolated and completely sequenced. Structural comparisons have revealed its relationship to other myosin genes. Nerve growth factor (NGF) is required for the differentiation of PC12 neuronal cells in vitro. Both cDNA clones and genomic clones representing a sequence induced 50-80 fold within 9 hours after treatment with NGF have been isolated and partially sequenced. The gene encodes a polypeptide of 85Kd.

IV. MACROMOLECULAR INTERACTIONS SECTION (Dr. Claude Klee, Chief)

This section is composed of three independent groups.

A. Dr. Cary Queen and coworkers: This group is studying the regulation of expression of the immunoglobulin gene family by attempting to answer two questions: (1) Why is synthesis of immunoglobulins restricted to cells of the B-lymphoid lineage; (2) How do these cells limit transcription to only one or a few immunoglobulin genes, while leaving other, similar immunoglobulin genes inactive? The approach to these questions is to insert a cloned, rearranged kappa light chain gene into a plasmid in various configurations, to transfect the plasmid into various types of cells, and to determine whether the transfected gene is transcribed. They have already shown that the complete kappa gene is transcribed after transfection into antibody-producing myeloma cells but not in nonlymphoid 3T3 or L cells. Hence the lymphoid cells appropriately regulate the kappa gene even when it is not in its usual chromosomal environment. By deleting different parts of the cloned gene, they have shown that certain sequence elements downstream of the promoter are necessary for gene transcription in myeloma cells. The downstream element has been localized to a 200 base pair region of DNA and shown to be a cell-specific enhancer. More recently they have shown that the promoter for an immunoglobulin kappa light chain gene is also strongly specific for lymphoid cells. Thus, both regulatory elements participate in cell specific gene expression.

B. Dr. Paul Wagner and coworkers: The goal of Dr. Wagner's group is to understand the role of the interaction of cytoplasmic myosins with actin filaments in the various motile activities of the cell. Whereas both smooth muscle and cyto-

plasmic myosins are regulated by phosphorylation of the 20,000 dalton light chains, which affects filament stability and stimulates the actin-activated ATPase, Dr. Wagner and his colleagues have now shown that their mechanism of activation is different. They have developed a method to separate myosin with two phosphorylated light chains from unphosphorylated and monophosphorylated myosin. Using this technique they were able to show that (1) thymus myosin is phosphorylated randomly. (2) the actin-activated ATPase of thymus myosin increases linearly with the extent of phosphorylation of the light chains; thus phosphorylation of one head of thymus myosin stimulates its ATPase independent of the phosphorylation of the second head. (3) ATPase activation is the result of an increase in the apparent affinity of thymus myosin for actin. (4) In contrast, gizzard myosin is phosphorylated in an ordered fashion and its activation requires phosphorylation of the two myosin heads. The activation of gizzard myosin is the result of a large increase in the maximum rate of ATP hydrolysis with a small change in the affinity of myosin for actin.

C. Dr. Claude Klee and coworkers: The major research goal of Dr. Klee and her coworkers is to understand the mechanism of regulation of cellular processes by calcium and calmodulin. Calmodulin is a ubiquitous calcium-binding protein which recognized changes in the intracellular concentration of calcium induced by external stimuli and transmits this information to cellular proteins whose activity is thereby altered. Stimulus-response coupling mediated by calmodulin involves several steps: (1) transitory increase in calcium concentration, (2) interaction of calcium with calmodulin which is accompanied by stepwise calcium-induced structural transitions, and (3) coordinated interaction with an activation of many calmodulin-regulated enzymes and proteins. Previous work in the laboratory showed that calcium binding to calmodulin is an ordered process. It has now been shown that the two calcium binding sites in the carboxyl-terminal half of the molecule are occupied first, and that the two low affinity sites are those in the amino-terminal half of calmodulin. Stepwise occupancy of these sites generates at least three different conformers. If one assumes that different enzymes recognize different calmodulin conformers then calmodulin could translate quantitative changes in calcium concentration into qualitatively different cellular responses. Thus, more recent efforts have been devoted to study of the interaction of calmodulin with several calmodulin-regulated enzymes such as cAMP phosphodiesterase, the calmodulin-regulated protein phosphatase, calcineurin, and three different protein kinases. Large tryptic fragments of calmodulin which encompass the amino (1-77) and the carboxyl (78-148) terminal halves of calmodulin preserve much of structure which they have in the native protein, bind calcium like calmodulin and undergo calcium dependent conformational transitions. These fragments also interact with the anti-calmodulin drugs, phenothiazines, in a calcium dependent manner. Whereas both peptides were known to activate phosphorylase kinase, neither fragment activates phosphodiesterase, calineurin, glycogen synthase kinase or myosin kinase. In spite of these differences in behavior, these fragments all interact with many enzymes when coupled to a solid matrix. Phosphodiesterase and calcineurin are retained on a column of fragment 78-148 coupled to Sepharose and are eluted with EGTA containing buffers. Calmodulin regulated kinase(s) is retained on the same column but only a low ionic strength. Fragment 1-77 interacts only with phosphodiesterase and cAMP-dependent protein kinase. Thus the interaction of calmodulin with these enzymes requires the integrity of different portions of the calmodulin molecule and the enzymes have different requirements for activation by or interaction with calmodulin. These observations have now been used to develop selective affinity chromatographic procedures to purify calmodulin-binding proteins.

These findings are in good agreement with last year's report that a covalent adduct of calmodulin with phenothiazine can act as an agonist or an atagonist depending upon the enzyme under study. Future efforts will be devoted to the isolation and characterization of the interaction sites of calmodulin with one of the enzymes under its control, the protein phosphatase, calcineurin. This enzyme which specifically dephosphorylates substrates of the cAMP-dependent kinase may play a major role in the calcium regulation of protein phosphorylation and thereby be a coupling factor between the two second messengers calcium and cAMP.

VI. NUCLEIC ACID ENZYMOLOGY (Dr. Maxine Singer, Chief)

Dr. Maxine Singer and her coworkers are studying two different classes of sequences that are highly repeated in primate genomes. The members of one class, the LINE-1 family, are interspersed in the genome. The members of the second class, centromeric satellite DNA, appear in long tandem arrays at centromeres. Emphasis is on the African green monkey genome and human DNA is also studied. The major primate family of highly repeated long interspersed DNA sequenced (called LINE-1, previously KpnI family) includes several thousand 6 kbp long units that terminate in an A-rich stretch. In addition there are more than 10^4 dispersed copies of truncated, rearranged and deleted LINE-1 segments in primate genomes. A 6 kbp sequence compiled from independently cloned and sequenced human and monkey LINE-1 subsegments contains open reading frames totalling about 3.5 kbp. The region containing the open reading frame is conserved in rodents. On the basis of these data these workers proposed that one or more LINE-1 family members may be functional genes and encode a protein. Therefore the existence of polyadenylated cytoplasmic RNA that might be messenger RNA for the putative genes was investigated. Such a transcript, which represents the sense-strand of the LINE-1 sequence, was detected in a human teratocarcinoma tissue culture cell line. The abundance of the transcript varies markedly with previously described variations in the phenotype of these cells. Thus, it is most abundant in non-contact inhibited cells with an embryonal carcinoma phenotype, is very scarce when the cell density is low, and is undetectable after the cells are induced to differentiate with retinoic acid. In a second approach to testing the hypothesis that one or more family members are functional genes, antibodies were prepared to synthetic peptides whose structure was predicted from the open reading frames. One of the antibodies precipitates a protein of an appropriate size from extracts of the teratocarcinoma cells. The immunoprecipitation is suppressed by the immunizing peptide. Thus, it appears likely that the LINE-1 family does include one or more functional genes and that these are expressed in cleavage stage embryos.

Certain satellite DNAs are characterized by long tandem repetitions of species specific sequences and centromeric location. Earlier work indicated that the organization of one such monkey satellite, deca-satellite, is highly polymorphic in individual members of the species and that the amounts of deca-satellite and α-satellite, the major monkey satellite, vary (independently) in individual genomes. In an effort to understand the maintenance of such extensive but variable DNA sequences, analysis of junctions between satellite and unique genomic sequence has been initiated. The rationale for this work is as follows. The fluid nature of centromeric satellite DNA sequences may be the consequence of important centromeric functions. Such functions are likely to be controlled by low copy number DNA sequences that neighbor satellite DNA. As a first step in testing this hypothesis, cloned genomic fragments containing junctions between satellite DNA and low copy number sequences must be isolated and characterized.

Using a specially designed strategy, three such monkey clones were isolated from a recombinant library. They contain the species-specific monkey deca-satellite joined to low copy number sequence. One of the clones has been characterized in detail. The relation between the cloned segment and genomic DNA was analyzed by DNA-blotting experiments and by isolation of homologous DNA sequences from an independent genomic library. Twenty or fewer copies of the low copy number sequence occur. Most copies are very similar, but polymorphic arrangements also occur. At least one copy of the low copy number segment and probably more are indeed linked to satellite DNA in the genome. The low copy number DNA segment was used as a probe against human and rodent genomic DNA. The sequence is conserved in all tested species. The monkey low copy number sequences were used to select cloned segments from a human genomic library. Most of the reactive clones (about 7 from one full library) also annealed to specific human centromeric satellite DNA. Thus both the low copy number sequence and its close association with satellite DNA is conserved in primates. Several observations suggest that these cloned "junction" segments contain recombinogenic segments. Thus, the clones are remarkably difficult to grow in their appropriate bacterial host; some isolated recombinant phage DNA's are rearranged, even within the vector sequences; at least some of the clones contain stretches of poly d(CA·GT), a sequence that is known to be recombinogenic.

VII. PROTEIN CHEMISTRY (Dr. Elbert Peterson, Chief)

The Protein Chemistry Section is made up of three independent research groups.

A. Dr. Michael Mage and coworkers: Continuing their development of methods for the specific isolation of immune cells and the study of their roles in cellular immune reactions, these investigators have investigated the subsets of $Lyt2^+$ T cells that are distinguished by the rat monoclonal antibody "B4B2" prepared by Dr. John Kung of the University of Texas. They found that both $B4B2^+$ and $B4B2^-$ lymphocytes were active in the in vivo graft vs. host reaction.

Also, in a collaborative study with Dr. P. Puccetti and colleagues at the University of Perugia, in vitro purified immune $Lyt2^+T$ cells had in vivo antitumor activity against intracerebral tumors in mice.

Since many tumors fail to elicit an effective immune response, "xenogenization" (induced mutation in tumor cells to increase their immunogenicity) is a potential approach to cancer immunotherapy. In a study of the immune response to such cells, a humoral response was obtained to a chemically xenogenized murine tumor cell line that was previously identified only through its ability to elicit a cellular immune response.

B. Dr. Warren H. Evans and coworkers: Myeloblast maturation factor (MMF) has been isolated from guinea pig serum in a highly purified form with an overall recovery of 25-30%. It migrates as a single band in both SDS and Ornstein-Davis gel electrophoretic systems and has an apparent molecular weight of 72,000-76,000, as determined by size exclusion chromatography and SDS gel electrophoresis. MMF activity is sensitive to digestion by protease, supporting the assumption that the activity resides in a protein molecule.

Human serum shows a strong MMF activity for guinea pig myeloblasts. Pure transferrin isolated from human serum in Dr. Evan's laboratory demonstrated potent

MMF activity, and three commercial preparations of human transferrin were also active. Studies in collaboration with Dr. E.A. Peterson, using displacement chromatography to purify and characterize MMF, indicate that whereas the activity of human serum is chromatographically coincident with transferrin, all of the activity of guinea pig serum is associated with a protein that is widely separated from transferrin chromatographically, though it closely resembles transferrin electrophoretically.

In studies aimed at the identification of molecular mechanisms responsible for arrested myeloblast maturation in the blast crisis stage of CML, Dr. Evans and his colleagues found that leukemic myeloblasts from that stage of his guinea pig CML model failed to respond to MMF under conditions in which this factor induces maturation in normal myeloblasts. Testing for defective receptors is being planned, and efforts to identify the genes in CML myelocytes that are responsible for the defective maturation program in these cells are continuing, in collaboration with Dr. O.W. McBride.

Using the HPLC assay previously developed for his studies of guinea pig granulocytes, Dr. Evans and his collaborators at the Walter Reed Army Medical Center have now found it possible to detect defects in the protein phenotypes of mature granulocytes from CML patients. These cells show major quantitative alterations in their differentiation protein patterns, most of them associated with granule protein markers. Present results indicate that a quantitative decrease in the height of one or more protein peaks in the HPLC pattern of the granulocytes from a CML patient can be used to predict the onset of the accelerated stage several months in advance of other clinical tests.

C. Dr. Elbert A. Peterson and coworkers: Technical improvements continue to be made in the synthesis, purification, and fractionation of the carboxymethyl-dextrans (CMDs) used in the ion-exchange displacement (IED) chromatography of proteins. These improvements have further simplified the overall procedure and include a means of reducing the absorbance at 280 nm substantially below that obtained previously, a matter of importance for applications on an analytical scale, where absorbance at this wave length is the primary means of delineating the protein profile.

A collaboration with Dr. A.R. Torres, a former co-worker in this laboratory, and his present associates at Yale University has demonstrated that two proteins differing in isoelectric point by only 0.1 pH unit can be completely separated by IED-chromatography in less than an hour on HPLC anion exchange columns that are half-saturated with these proteins. Such resolution was achieved with a protein load of 85 mg per ml of column bed. This remarkable capacity is of great importance to the use of HPLC columns for preparative scale purifications in both industry and the laboratory.

A method employing IED-chromatography has been developed for the isolation of the myeloblast maturation factor (MMF) of Dr. Evans from guinea pig serum. The protein associated with the activity can be obtained almost pure, contaminated by only traces of other proteins according to Ornstein-Davis gel electrophoresis, in a single pass at pH 7. Rechromatography on the same column at pH 8.2 with spacers of somewhat higher affinity provides a product that yields a single band in gel electrophoresis. Chromatography under sterile conditions permits all of the fractions to be used directly in Dr. Evans' assay, which involves incubation with myeloblasts for several days; the 10,000-dalton CMDs in the fractions do not

affect the cells adversely. In the guinea pig serum, protein at the transferrin position was inactive; the activity was all in fractions that contained a protein that resembled transferrin in electrophoretic position but was widely separated from it chromatographically. In human serum, however, the MMF activity was found in the transferrin peak, as had earlier been determined by Dr. Evans, and it appeared nowhere else in the chromatographic fractions.

In collaboration with Drs. M. Belew and J. Porath, the behavior of guinea pig serum proteins on several new adsorbents brought from Uppsala, Sweden, has been investigated. The results indicate that preliminary passage of the serum through a tandem series of these should make possible the isolation of pure MMF in a single IED-chromatography as well as eliminate the need for dialysis of the serum.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00333-22 LB

PERIOD COVERED

October 1, 1984, to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical Basis for Defective Differentiation in Granulocytic Leukemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

W. H. Evans	Research Chemist	LB	NCI
E. A. Peterson	Chief, Protein Chemistry Section	LB	NCI
S. Wilson	Biologist	LB	NCI
M. Mage	Immunochemist	LB	NCI
W. McBride	Chief, Cellular Regulation Section	LB	NCI
R. Balachandran	Visiting Associate	LB	NCI

COOPERATING UNITS *(if any)*

Hematology, Oncology Section, Walter Reed Army Medical Center

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The main thrust of this work is to develop biochemical methods for the early diagnosis of granulocytic leukemia and methods for inducing leukemic cells to develop some or all of their functional properties as a means of partially or completely restoring host defense mechanisms in leukemia patients. Work is first aimed at establishing which of the many biochemical steps involved in normal granulocyte differentiation are controlled by humoral regulators. The results will be compared with those obtained from similar studies on leukemic cells at corresponding stages of maturity in order to determine the nature and potential reversibility of the arrested differentiation steps. Biochemical analyses are carried out on mature and immature granulocytes isolated from blood and bone marrow and the effects of external cell regulators on granulocyte differentiation, as measured by changes in the synthesis of specific cellular components, are studied in a defined culture system previously developed in this laboratory. Possible relationships between transforming genes in leukemic myeloblasts and factors involved in the regulation of normal granulocyte differentiation are under investigation.

Project Description

Methods Employed:

Leukocytes are isolated from bone marrow, blood and inflammatory exudates by a variety of methods. Subcellular fractions are prepared by differential centrifugation. Proteins extracted from leukocytes and serum proteins are fractionated by column chromatography, high performance liquid chromatography (HPLC), and gel electrophoresis. Glycolipids are separated by thin layer chromatography and analyzed by gas-liquid chromatography and mass spectrometry. Immunological reagents, including fluorescent antibodies, are prepared against granulocyte proteins and used to follow their formation during granulocyte maturation. Stationary tissue culture methods are employed to study the synthesis of specific proteins from radioactive precursors. The distribution of radioactive proteins in electrophoretic gels is determined by slicing the gels in a gel fractionator and counting the slices in a liquid scintillation counter. Densitometric patterns of gels stained for glycoproteins are obtained by scanning the gels in a spectrophotometer equipped with a gel scanner accessory. Genetic analyses are carried out using transfection assays, agarose gel electrophoresis-Southern blot analyses of normal and leukemic DNA fragments obtained by restriction endonuclease cleavage and dot blot hybridization of cellular RNA with ^{32}P-labelled viral oncogene probes.

Major Findings:

I. Purification of Myeloblast Maturation Factor (MMF)

We showed previously that normal granulocyte maturation from myeloblasts is dependent on a humoral regulator in normal serum which we call myeloblast maturation factor (MMF). During the past year our work has been aimed at characterizing and purifying MMF from normal serum. This work has been greatly facilitated by the application of our newly developed quantitative biochemical micro-assay for MMF described previously. Using this new assay and conventional chromatographic methods we have isolated MMF from guinea pig serum in a highly purified form with an overall recovery of 25-30%. The purified material migrates as a single band both in the SDS gel and the Davis-Ornstein gel electrophoretic systems and has an apparent molecular weight of 72,000-76,000 as determined by size exclusion HPLC and by the SDS gel electrophoretic method. MMF is sensitive to protease digestion but is little affected by neuraminidase treatment suggesting the activity is associated with a protein and that sialic acid groups are not involved in the active site of this factor.

The physical properties and relative abundance of the purified factor in normal guinea pig serum suggested that MMF was either transferrin or a transferrin-like protein. To test this hypothesis we purified transferrin from a known source, namely, human serum and assayed this protein for its ability to induce the maturation of guinea pig myeloblasts to mature granulocytes *in vitro*. Our results show that the transferrin fraction of human serum is a potent source of MMF. Preliminary results indicate that at least three commercial preparations of human transferrin also have MMF activity. These results strongly indicate that human MMF is transferrin and that the MMF

activity of this protein is not species specific. Studies are now underway to determine the effect of human transferrin on the maturation of human myeloblasts in vitro.

Studies in collaboration with Dr. E.A. Peterson, using displacement chromatography to characterize and purify MMF, indicate that whereas the activity of human serum is chromatographically coincident with transferrin, all of the activity of guinea pig serum is associated with a protein that is widely separated from transferrin chromatographically, though it closely resembles it electrophoretically.

II. Molecular Basis of Defective Myeloblast Maturation in CML Blast Crisis

Another important goal of our work is to attempt to identify the molecular mechanisms responsible for arrested myeloblast maturation in the blast crisis (BC) stage of human CML. For this work we are currently using leukemic myeloblasts from the blast crisis stage of our recently developed guinea pig leukemia model for human CML. Our cell culture studies indicate that these leukemia myeloblasts fail to respond to MMF under conditions in which this factor induces maturation in normal myeloblasts. Maturation regulation in leukemic cells could be defective at several levels. One obvious hypothesis which is testable with our maturation assay is that MMF fails to bind to target cells in a normal manner due to a defective receptor for this regulator.

Work continues on the problem of attempting to identify the transforming genes in CML myeloblasts that are ultimately responsible for the defective maturation program in these cells. This work is being carried out in collaboration with Dr. O.W. McBride and is described in his Annual Report.

III. Detection of Defects in the Phenotypic Protein Patterns of Human CML Granulocytes.

Using the HPLC assay developed for our studies of guinea pig granulocytes described previously we now find that it is possible to detect defects in the protein phenotypes of mature granulocytes from CML patients. A paper describing this work has recently been accepted for publication in the journal of the National Cancer Institute. Briefly, we find that relative to normal granulocytes, mature granulocytes from CML patients show major quantitative alterations in their differentiation protein patterns. Most of these defects are associated with granule protein markers. Each patient in the chronic stage of CML appeared to have a constant and characteristic abnormal protein phenotype but this phenotype varied considerably between individual patients. These findings suggest that the synthesis of differentiation proteins in normal granulocytes is more tightly controlled than that in CML granulocytes. In this connection it is interesting to note that the size of the Ph^1 chromosome fragment in CML cells also varies considerably among CML patients. Variability in the rearrangement and expression of various oncogenes in CML granulocytes has also been observed by others. Taken together, these observations suggest that the phenotypic variation we have observed in CML granulocytes could be a reflection of these underlying genotypic variations.

Studies are now underway to determine whether the HPLC assay developed in our lab can be used to assess the progression of human CML from the relatively benign, stable phase to the more acute, accelerated phase. Our results, thus far, indicate that a quantitative decrease in the height of one or more protein peaks in the HPLC protein pattern of the granulocytes from a CML patient can be used to predict the onset of the accelerated stage several months in advance of other clinical tests. CML patients at Walter Reed Army Hospital are now being monitored routinely under an experimental protocol designed to further evaluate this assay as a prognostic indicator in CML.

Significance to Cancer Research:

Granulocytic leukocytes are produced in the bone marrow by a complex, multistage process of cell differentiation whereby proliferating, nonphagocytic, precursor cells are converted to nonproliferating phagocytic cells that play a crucial role in the body's defense against microbial invasion. Granulocytic leukemias can be thought of as diseases in which this program for differentiation is arrested at various stages, resulting in the overproliferation of immature cells lacking, in varying degrees, the capacity to carry out their normal function in the body's defense against infections. At present, the mechanisms involved in the initiation and modulation of the various steps in the differentiation program are poorly understood. Our research is aimed at identifying the humoral factors and clarifying the molecular processes that control the appearance of specific subcellular components during granulocyte differentiation. Such information should be useful in developing therapeutic approaches for reversing the arrested differentiation of leukemic cells.

Proposed Course of Research:

Our research plans are as follows:

(1) To further characterize the MMF found in normal human serum and to determine whether human CML and AML myeloblasts are defective with respect to their response to this factor as has been found with leukemic myeloblasts from guinea pigs.

(2) In collaboration with Dr. Michael Mage we will attempt to isolate and purify the MMF receptor in normal myeloblasts and determine whether or not this receptor is defective in leukemic myeloblasts. For this work we will use isotope-labelled MMF and antibodies to MMF to detect the presence of MMF receptor on intact cells or in various detergent lysates. If detected, this receptor will be purified using standard affinity separation procedures. We will then isolate the MMF receptor from leukemic myeloblasts and determine whether it differs markedly in composition from the normal receptor. If the receptor mechanism involves a defective protein, then it would be important to determine whether the gene coding for this receptor is related to any of the known oncogenes. This working hypothesis for a possible mechanism of tranformation in CML has a precedent in epidermal cellular transformation. In transformed epidermal cells, the receptor protein for epidermal growth factor (EGF) has been found to be defective and the defect has been related to a known oncogene (V-erb-B) which codes for the EGF receptor.

(3) The availability of our guinea pig model for human CML opens up many new possibilities for studies of the molecular genetics of this disease, especially since some of these studies cannot be carried out in humans. We are particularly interested in determining whether it is possible to transform normal guinea pig myeloblasts with genetic material isolated from leukemic myeloblasts. For this purpose we plan to develop a rapid assay for detecting transformed myeloblasts in vitro, since such an assay is currently not available. We will explore the possibility of using the selective effect of phytohemagglutinin stimulation on leukemic myeloblast colony formation in soft agar cultures as a means of detecting normal cells which have been tranformed by leukemic cell DNA. DNA and RNA extracted from normal and leukemic myeloblasts will be screened further with a variety of oncogene probes in an attempt to detect altered proto-oncogenes in leukemic myeloblasts. (See Dr. O.W. McBride's Annual Report for further details of this approach).

(4) To further develop the use of HPLC as a method for phenotypically characterizing the degree of differentiation of purified populations of normal and leukemic granulocytes from guinea pigs and humans. This rapid screening method might be useful in classifying or staging AML and CML patients for various types of therapy.

(5) To investigate various potential agents for inducing leukemic cells to mature either partially or completely in vitro. Such agents could serve as attractive adjuncts, or in some cases, as alternatives to cytotoxic therapy, for they would inhibit the expansion of the leukemic population while leaving normal granulocytes unharmed.

Publications:

Evans, W.H., Miller, D.A., Pearson, J.W., Smalley, R.V., Moore, D.E., and Terebelo, H.R.: Busulfan versus cyclophosphamide treatment in the early and late stages of granulocytic leukemia in guinea pigs. Leukemia Research 8: 1037-1042, 1984.

Evans, W.H. Bednarek, J.M., Alvarez, V.L., Wright, D.G., Terebelo, H.R., and Taylor, G.: Direct analysis of differentiation proteins in normal and leukemic human granulocytes by high performance liquid chromatography. J. Natl. Cancer Inst. (in press).

Appendix: NIH Contract No. 1-60-25423

Funding: General NCI Contract for FCRC - No individual project funding breakdown available.

Man Years Purchased: 1

Major Findings:

During the past year this contract was used primarily for the *in vivo* and *in vitro* maintenance of cells from our guinea pig model for human CML. These cells were also used for our studies aimed at identifying the molecular mechanisms responsible for arrested myeloblast maturation in the blast crisis stage of CML.

As an addendum to our previous study on the use of cyclophosphamide in the treatment of this leukemia we observed that this drug produces the same high cure rate when administered in the intermediate stage of the disease as it did in the previously studied early stage.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00366-15 LB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(60 characters or less. Title must fit on one line between the borders.)*
Structure and Expression of Endogenous Retroviral Elements

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

E. L. Kuff	Chief, Biosynthesis Section	LB	NCI
K. K. Lueders	Chemist	LB	NCI
A. Feenstra	Visiting Fellow	LB	NCI
Z. Grossman	Visiting Fellow	LB	NCI

COOPERATING UNITS *(if any)*
E. Leiter, Jackson Laboratory, Bar Harbor, ME
K. Moore, DNAX Research Institute, Palo Alto, CA
K. Ishizaka, Johns Hopkins School of Medicine, Baltimore, MD

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Biosynthesis Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	1.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous studies in this project have identified the intracisternal A-particle (IAP) genes as a genetically unique type retrovirus-like element extensively reiterated in the genomes of mice and some other rodent species. The past year has seen a surprising development in which IAP genes have been linked to a class of soluble immunoregulatory molecules that can potentiate or suppress Ig production in target B cells. Drs. K. Moore and K. Ishizaka succeeded in obtaining a cDNA clone which coded for 2 related polypeptides that enhanced production of IgE in mouse B-cells in vitro. Comparison of their nucleotide sequence with that obtained in our laboratory for an authentic endogenous IAP gene firmly established that the cDNA represented a modified IAP element coding for type of gag-pol fusion protein. Antiserum previously prepared by us against purified IAP structural proteins was shown to bind the active immunoregulatory polypeptides produced both by the cDNA clone and by normal I-lymhpocytes.

In a continuing study with Dr. E. Leiter, on the possible role of IAP antigen expression in pathogenesis of insulin-dependent diabetes in mice, we have shown that IAP mRNA and proteins were induced in isolated pancreatic islets incubated in a high glucose medium and that both the basal and glucose-induced levels were significantly higher in islets from normal mice susceptible to the diabetogenic action of the db gene than in those from resistant strains. We postulate that beta cells carrying IAP-related antigen(s) on their cell surface may be subject to autoimmune attack.

Project Description

Objectives:

To study the regulation of endogenous viral gene expression in relationship to normal development and cellular function, neoplastic transformation and other disease states.

Methods Employed:

Culture of animal and bacterial cells; recombination and cloning of specific eukaryotic and viral DNA sequences in plasmids, lambda phage and retrovirus vectors; analysis of DNA components by restriction endonuclease cleavage, electrophoresis, and blot hybridization; chromosomal localization of specific DNA sequences by in situ hybridization; electron microscopy of DNA heteroduplexes; nucleotide sequencing by the chemical procedure of Maxim and Gilbert and the dideoxy method of Sanger; chromatographic and electrophoretic analysis of cellular and viral proteins; radio-immunoassay and immunoprecipitation of specific antigens; immunofluorescent staining; transfection of cultured cells with recombinant plasmids formed from expression vectors.

Major Findings:

Intracisternal A-particle (IAP) genes represent a distinctive class of endogenous retrovirus-like elements that are extensively reiterated in the cellular DNA of Mus musculus and certain other rodent species. The family of mouse IAP genes, about 1000 per haploid genome, includes structural variants with deletions and insertions at characteristic positions. During the past year we have been concerned particularly with the properties and possible functional roles of peptides produced by full-sized and variant IAP genes. A novel link has been established between IAP genes and a type of T-cell derived factor involved in differentiation of B-lymphocytes.

I. Relationship Between IAP Genes and Immunoregulatory Factors

T-lymphocytes secrete a variety of factors involved in the activation of immunoglobulin (Ig) production by target B-cells. Among them are polypeptides which act to modulate Ig synthesis in the differentiating B-lymphocytes. These polypeptides are specific for each Ig type. The secreted factors can be bound to affinity columns of the Ig (they are designated Ig-binding factors or Ig-BF), and subsequently eluted in active form. Dr. Kimishiga Ishizaka at Johns Hopkins University School of Medicine has studied the IgE-BF of rats and mice over many years. These factors are glycoproteins which, depending upon the extent of glycosylation, can have either potentiating or suppressing effects on IgE production in B-lymphocytes. The secreted proteins share antigenic determinants with Fc_E receptors on the lymphocyte surface.

Because the Ig-BF are normally produced in minute amounts, Drs. Ishizaka and Kevin Moore of DNAX Research Institute set out to obtain cDNA clones that could code for the IgE-BF of rats. They worked with a T-cell hybridoma obtained by fusion of a normal rat T-cell and a mouse T-lymphoma. This hybridoma, 23B6, produced soluble IgE-BF when stimulated by rat IgE. The

factor(s) had suppressing effects on IgE production when applied to target B-cells in an in vitro assay. Several cDNA clones were obtained which, upon transfection into a monkey kidney cell line (COS), coded for secreted polypeptides that bound to IgE. Most of these IgE-BFs were without effect on IgE production in the B-cell assay system. One, however (the product of cDNA clone 8.3) had marked potentiating effects on IgE production. The 3300 base pair nucleotide sequence of clone 8.3 was determined in full.

Drs. Moore and Ishizaka obtained preliminary evidence for a nucleotide sequence homology between clone 8.3 and the mouse IAP genes, and at this point established a collaborative relationship with our laboratory. Comparison of the clone 8.3 sequence with our data on an authentic genomic IAP gene established that the cDNA clone represented a variant mouse IAP gene carrying a major deletion in the gag and pol regions. We showed that the 556 base pair open reading frame in clone 8.3 consisted of 432 codons derived from the IAP gag coding region and 124 codons derived from the 3' end of pol. Thus, the secretory product had the form of an IAP-related gag-pol fusion product. On this basis, we suggested that antisera directed against the authentic IAP gag protein might react with the IgE-BF coded by the cDNA clone. This proved to be the case. Several of our rabbit antisera reacted not only with the clone 8.3 product but also with the IgE-BFs produced by the 23B6 hybridoma and by normal rat and mouse T-lymphocytes in vivo. Dr. Moore has also studied several of the cDNA clones that coded for Ig-BFs with no apparent influence on B-cell differentiation. These clones also represent modified IAP genes, but they are structurally distinct from clone 8.3.

II. IAP

This area is of enhanced interest in view of the sequence relationship between the IgE-binding factors and the major IAP structural protein (p73). As described in last year's Annual Report, antisera against p73 immunoprecipitate additional protein species from extracts of metabolically labelled mouse tumor cells and normal thymocytes. Chief among them are polypeptides with molecular weights of 114 to 120 kilodaltons. These are produced by variant IAP gene forms with deletions encompassing the 3' portion of the gag (p73) and 5' portion of the pol coding regions. The p114-120 proteins represent gag-pol fusion products, analogous to but differing in detail from the IgE-BF produced by clone 8.3. We are investigating several questions with regard to these proteins, using mouse normal thymocytes and T-cell lymphomas as experimental systems.

A. Intracellular location

The p114-120 proteins are membrane-associated but easily solubilized with neutral detergents. This is in contrast to p73 which is tightly aggregated in the particle cores and requires strong denaturing agents for solubilization. We are particularly interested in the possible location of p114-120 proteins at the cell surface. We have recently observed cell surface immunostaining of the 23B6 rat-mouse T-cell hybridoma using antisera against IAP p73. Previously, we reported a patchy localization of IAP-related antigen at the surfaces of mouse pancreatic beta-cells induced for IAP expression by high blood glucose levels (see Section 3

below). The p114-120 polypeptides are the most abundantly synthesized IAP-related proteins in both cell types and are candidates for the surface-associated antigens. Identification of these components may be achieved by selection iodination of cell surface proteins followed by immunoprecipitation of labelled proteins with IAP-specific antiserum.

B. Cloning of IAP Genes Coding for p114-120 Polypeptides

We are preparing a cDNA library from the polyA$^+$ RNA of thymuses from strain C58/J mice. Thymocytes of this strain synthesize very little p73 but relatively high levels of the larger polypeptides. A 5.4 Kb RNA species is the strongly predominant IAP transcript. The library is being prepared in an Okayama-Berg vector which will permit expression of IAP-related cDNA clones transfected into transformed monkey kidney (COS) cells. It is anticipated that the cDNA clones will provide detailed information about the structure of the p114-120 polypeptides of normal thymocytes. These clones will be compared with the IgE-BF producing clones prepared in the same vector by Dr. Moore (and provided to us). One of the cDNA clones isolated by Dr. Moore codes for a secreted 60 Kd IgE-BF but has an open reading frame large enough for a 116 Kd polypeptide. This clone has precisely the deleted structure we envisaged for the variant IAP genes that code for the p114-120 polypeptides.

C. Do Other IAP Genes Code for Soluble Binding Factors and Fc Receptors for Other Types of Immunoglobulins?

The IgE-BFs are antigenically related to the Fc_E receptors on the lymphocyte surfaces. Therefore, it is likely that the receptors may also be coded by IAP-related genes. Soluble binding factors and Fc receptors exist for the other immunoglobulin classes. These receptors have not been cloned and their structure is not known. A rat monoclonal antibody against a mouse Fc_γ receptor is available and we will test this against IAP protein in an ELISA assay. We are also preparing to test for affinities between IAP-related proteins and the major immunoglobulin types.

III. The Role of IAP Gene Expression in the Pathogenesis of Genetically Determined Diabetes in the Mouse.

In mice of certain genetic backgrounds, e.g. C57BL/Ks and CBA, the homozygous db gene produces a sequence of events leading from high blood glucose to an autoimmune-mediated beta-cell necrosis and insulin-dependent diabetes. We have collaborated with Dr. E. Leiter in studying the possible role of IAP protein expression in this process. As reported last year, IAPs and membrane-associated IAP-related antigens were found by immunoelectron microscopy to be constitutively expressed in beta-cells of diabetes-susceptible mice and to increase in cells from mice with elevated blood glucose levels. Since then, we have examined IAP gene expression in the islets of normal animals from susceptible and resistant strains when these islets were isolated and cultured in the presence of normal or high glucose levels.

Synthesis of immunoprecipitable IAP related protein was significantly enhanced in islets from normal C57BL/Ks and CBA mice in response to high glucose

medium, and IAP-specific RNA was increased more than 8-fold in the glucose-induced CBA islets. The basal and glucose-enhanced levels of IAP protein expression were both much lower in islets from normal mice of the diabetes-resistant C57BL/6 strain. We propose that in the beta-cells of genetically susceptible mice, one or more IAP genes are strongly glucose-inducible and that high blood glucose, which is the primary phenotype of the db gene, leads to increased expression of IAP proteins both within the cells and at their surfaces. In this model, IAP-related antigens are responsible for or contribute importantly to the autoimmune susceptibility of the affected beta cells. Establishment of such a direct link is the focus of present studies.

Significance for Cancer Research

As endogenous transposable elements, IAP genes may have a role in transformation or tumor progression. Activation in mouse tumors of IAP genes coding for immunoglobulin regulatory factors could have important effects on the host immune system.

Proposed Course of Research:

Over the next year, we will concentrate on studies related to the structure and possible function of variant IAP genes and their polypeptide products. The relationship between IAP genes and certain immunoregulatory proteins will be further studied.

Publications:

Lueders, K.K., Fewell, J.W., Kuff, E.L., and Koch, T.: The long terminal repeat of an endogenous intracisternal A-particle gene functions as a promoter when introduced into eucaryotic cells by transfection. Mol. Cell. Biol. 4: 2128-2135, 1984.

Kuff, E.L., and Fewell, J.W.: Intracisternal A-particle gene expression in normal mouse thymus tissue: Gene products and strain-related variability. Mol. Cell. Biol. 5: 474-483, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00375-23 LB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Homogeneity and Structure of Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

E.A. Peterson	Chief, Protein Chemistry Section	LB	NCI
M. Belew	Guest Researcher	LB	NCI
J. Porath	Fogarty Scholar		
A.R. Torres	Department of Laboratory Medicine, Yale University		

COOPERATING UNITS *(if any)*
Department of Laboratory Medicine, Yale University
University of Uppsala, Sweden

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Methods for the fractionation and analysis of proteins are developed and applied to the purification of specific proteins for the study of their function and structure. Ion-exchange displacement (IED) chromatography is being developed for the fractionation of macromolecules and particles of biological interest, employing polyanions differing in number of charges per molecule as displacers. The procedure is particularly advantageous when large amounts of source material must be used to obtain sufficient amounts of a minor component, since the resolving power of the system can be focused on the narrow range of affinity represented by the protein of interest and its nearest neighbors. However, it is also applicable to ion-exchange HPLC. Recent efforts have been directed toward the simplification of the preparation of narrow-range displacers. Application of the method to the isolation of the myeloblast maturation factor (MMF) of W. Evans (Project Number Z01 CB 00333-22 LB) from guinea pig serum has provided an electrophoretically homogeneous protein after two stages of displacement chromatography, but its chromatographic fractions were not uniform with respect to specific activity of MMF. An exploration of the binding of serum proteins to several metal chelate and hydrophobic adsorbents suggests that chromatography on some of these before IED-chromatography will simplify and improve the purification.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To develop methods for the separation and analysis of proteins that will overcome present limitations and to apply these methods to the purification of specific proteins for the study of their functions.

Methods Employed:

Protein fractions separated by ion-exchange displacement (IED) chromatography are evaluated by gel electrophoresis, using both denaturing and nondenaturing conditions. Carboxymethyldextrans (CM-Ds) having a variety of degrees of substitution are prepared by reaction of dextran with chloroacetate in alkaline solution.

Major Findings:

A. The fractionation of individual preparations of 5000-dalton CM-D obtained by complete reaction at 80°C of reagent-limiting reaction mixtures has shown them to be more heterogeneous than expected. The higher temperature now used may result in substitution at positions that were largely unaffected at the low temperatures previously used for making 10,000 dalton CM-Ds or there may be differences in the branching of these dextrans that give rise to differences in reactivity. Only two preparations are now needed to provide the full-range series of narrow-range CM-D fractions. In fact the most useful portion of that range can be obtained with a single preparation. This is substantially simpler than the multiple CM-D preparations of successively higher average affinity that were used in earlier fractionations of CM-D, and it eliminates any need for introducing heterogeneity in the reaction product by continuous or intermittent withdrawal during reaction. However, this high level of natural heterogeneity precludes the possibility of preparing a short series having a restricted range of affinity, such as would be desirable for specific applications. Also, because of inherent limits on concentration and volume limitations arising from the necessity for stopping the application of a given preparation to the fractionating column in time to allow all of its lowest-affinity component to emerge before the next higher component, application of the full range of activities in only one or two segments restricts the total load that can be applied. The capacity of the fractionating adsorbent (DE-23) is very much higher for CM-D(H) than for CM-D (Na) but applying the free-acid form results in a critical increase in resistance to flow, and final displacement of the highest affinity CM-D with NaOH must be gradual to avoid a very sharp increase in effluent concentration that can result in shearing of the mobile phase, with consequent mixing. Both of these technical difficulties have been brought under control.

B. Continuing an exploration of the effectiveness of CM-D displacers for the separation of proteins on micro HPLC columns (see report for 1982-83), a collaborative investigation of the capacities of three commercially available HPLC ion-exchangers for the resolution of β-lactoglobulins A and B was undertaken with Dr. A.R. Torres, a former co-worker in this laboratory, and his present associates at Yale University. These proteins are genetic variants

that differ in isoelectric point by about 0.1 pH unit, so the separation is a relatively demanding one. Nevertheless, complete separation was accomplished with a narrow range CM-D in less than an hour on columns that were 50% saturated with protein, and this does not represent the absolute limit. The three adsorbents varied widely in the amount of protein required to saturate them but for the one with the highest capacity the results indicate that high resolution can be achieved with a protein load of more than 85 mg per ml of column bed. This high capacity is of great importance to the use of HPLC columns for preparative scale purifications.

C. The development of an ion-exchange displacement (IED) method for isolating the myeloblast maturation factor (MMF) of Dr. Evans from guinea pig serum has been undertaken because a procedure suitable for adaptation to a larger scale of operation is needed. Also, this application demonstrates the feasibility of using CM-Ds in the 10,000-dalton range without adversely affecting the cells with which the fractions are incubated for days in the in vitro biological assay. By introducing a small 0.45 μm filter between the pump and the column before sterilizing the latter with sodium azide, the need to sterilize the several CM-D solutions separately before use was obviated, and the fractions (collected in sterile tubes) could be used directly in the assay. When 2 ml of dialyzed normal guinea pig serum was applied to a 7-ml column of DEAE-Sephacel equilibrated with sodium phosphate at pH 6.9 and followed with CM-D spacers and a final displacer, the MMF activity emerged between CM-Ds having RPV values (previously $1/V_p$; see report for 1979-80 for this measurement) of 6.4 and 7.6. In gel electrophoresis employing the Ornstein-Davis system, the protein associated with the activity appeared at very nearly the same electrophoretic position as the transferrin of this serum but was chromatographically well separated from it. The gel showed a series of overlapping, physically similar proteins beginning with transferrin and extending across the entire chromatogram. The MMF activity was found in several fractions beyond the albumin peak, contaminated with only faint traces of other proteins. The transferrin peak was inactive, as were other components of this series on either side of the active fractions. (In human serum the activity was found in the transferrin peak and nowhere else.) Several if not all of the proteins in this series are red, as judged by pink color on the column, contributing to the impression of physical relatedness. A change in the selection and relative quantities of the spacers caused the active material to emerge at a much higher concentration but with larger amounts of impurities. When these fractions were adjusted to pH 8.2 and rechromatographed on the same column equilibrated with Tris·HCl buffer at that pH, the impurities moved to different positions, leaving the MMF-associated protein apparently pure, according to gel electrophoresis. Porelimit gradient gel electrophoresis showed a limited microheterogeneity with respect to molecular weight.

Dr. Makonnen Belew is a Guest Researcher in this laboratory, having accompanied Dr. Jerker Porath, a Fogarty Scholar-in-Residence, from Uppsala, Sweden. They have brought with them a number of adsorbents with affinities for certain groups of proteins. Trial applications in this laboratory have indicated that the MMF activity of guinea pig serum that is passed through a tandem series of these adsorbents is bound to certain zinc or copper chelate groups. Several other proteins are also adsorbed but not those that are most difficult

to separate from MMF by displacement chromatography. This offers a simple preliminary fractionation that should facilitate purification by ion-exchange displacement.

Significance to Cancer Research:

Displacement chromatography of proteins promises to be of value at any scale of operation and therefore has significance to all research that involves the isolation of such substances. The high capacity, resolving power, and convenience of these systems offer to expedite the recognition and isolation of minor protein components such as regulating factors and marker proteins of interest in disease. The anticipated development of a systematic, general procedure for the purification of nonhistone nuclear proteins that participate in the regulation of the transcription of genetic information would be of substantial significance to cancer research since defective control of these processes appears to be involved in cancer.

Proposed Course of Research:

Development of displacement systems for the separation of nonhistone nuclear proteins will be continued. A large scale isolation of HMG-1 and HMG-2 will be undertaken and the LMG proteins will be fractionated into several groups with selected narrow-range spacers.

Displacement chromatography will be applied to the purification of enzymes and other proteins of interest to members of this Laboratory. Preliminary trials on tissue cytosols have been very promising. Possible application in the fractionation of leukocyte granules and cell populations will be explored, using appropriate adsorbant matrices. Also the narrow-range CM-Ds will be further tested as spacers in analytical separations on HPLC ion-exchange columns in the hope that they will improve resolution as well as eliminate the need for gradients.

Publications:

Torres, A.R., Dunn, B.E., Edberg, S.C., and Peterson, E.A.: Preparative high-performance liquid chromatography of proteins on ion-exchange columns with carboxymethyldextrans as displacers. J. Chromatogr. 316: 125-132, 1984.

Torres, A.R., Krueger, G.G., and Peterson, E.A.: Purification of Gc-2 globulin from human serum by displacement chromatography: A model for the isolation of marker proteins identified by two-dimensional electrophoresis. Anal. Biochem. 144: 469-476, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00945-12 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Factors Regulating the Synthesis of Collagen in Normal and Transformed Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

B. Peterkofsky	Research Chemist	LB	NCI
G. Majmudar	Visiting Associate	LB	NCI
T. Bird	Visiting Fellow	LB	NCI
I. Oyamada	Visiting Fellow	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.4	2.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The objective of one of our projects is to determine the mechanism by which vitamin C (ascorbic acid) controls connective tissue metabolism. Previously we showed that decreased collagen synthesis in parietal bone and cartilage of scorbutic guinea pigs was directly related to the extent of weight loss during the third and fourth week of scurvy, rather than to defective proline hydroxylation. Our current studies show that synthesis of another major component of cartilage extracellular matrix, proteoglycan, also is decreased and that synthesis of collagen and proteoglycans appears to be coordinately regulated. These, and other results, suggest that ascorbate deficiency indirectly produces these effects by inducing anorexia, which leads to a chronic fasting state. Acute fasting for 96 hr with ascorbate supplementation causes a similar coordinate reduction in collagen and proteoglycan production. Decreased collagen production in both bone and cartilage of acutely fasted and scorbutic animals is not due to an increase in degradation but to decreased synthesis caused mainly by a reduction in the levels of procollagen mRNAs.

In a second study, we have found that in a nitroquinoline oxide transformant of Syrian hamster embryo fibroblasts (NQT-SHE), there is almost complete supression of synthesis of type I procollagen, the major product of the parent cells. This suppression is correlated with the absence of mRNA for pro α_1I collagen polypeptide. In addition, synthesis of two previously undescribed types of collagen is induced. Both of these molecules appear to have a procollagen type of structure. Each is composed of a single subunit with a pepsin-resistant helical region having a typical repeating glycyl tripeptide sequence susceptible to bacterial collagenase, plus pepsin-sensitive noncollagenous regions. Current efforts are aimed at characterizing these collagens.

Project Description

Objectives:

To elucidate the basic mechanisms of collagen synthesis and secretion and to investigate the factors which regulate these processes in normal and transformed cells.

Methods Employed:

I. Proteins of isolated tissues, cultured cells or cell-free systems are labeled with ^{3}H or ^{14}C-proline. The proteins are precipitated with trichlo ro acetic acid, redissolved and then assayed for radioactivity in collagen and noncollagenous proteins. The method involves specific digestion of collagen in a mixture of proteins using highly purified collagenase and was developed in our laboratory. The relative rate of collagen synthesis can be calculated from data obtained by this method and the specific rate of synthesis in tissues or cells is calculated by determining the DNA content and expressing the rate as cpm/μg DNA. The proteins of the cell and medium fractions of cultured cells are analyzed separately in order to study secretion. Morphological changes in cultured cells are recorded by polaroid photomicroscopy.

II. The level of proline hydroxylation in collagen of cells or tissues is measured by a dual labeled proline method which was devised in this laboratory and eliminates the necessity for hydrolysis. To measure the level of lysine hydroxylation, cells or tissues are labeled with radioactive lysine, and hydrolyzed collagenase digests are chromatographed on Dowex-50 in 2N HCl, which separates lysine and hydroxylysine.

III. Prolyl and lysyl hydroxylases are measured by $^{3}H_2O$ release from ^{3}H-proline or ^{3}H-lysine labeled unhydroxylated collagen prepared by incubating chick embryo frontal bones with the labeled amino acid in the presence of the iron chelator α, α-dipyridyl, which inhibits hydroxylation.

IV. Collagen types synthesized in cultured cells or tissues are determined by analysis of ^{14}C-or ^{3}H-proline labeled, denatured collagen using electrophoresis in sodium dodecyl sulfate-polyacrylamide slab gels. Disulfide bonds are detected by running samples with and without dithiothreitol and observing alterations in the positions of α chains. Radioactive proteins are detected by fluorography.

V. Cyclic 3', 5' adenosine monophosphate(cAMP) is measured by a competitive binding assay. Cells are extracted with trichloroacetic acid, the cAMP purified by passing the extract through a cation exchange resin column and the extent of binding to a purified cAMP-dependent protein-kinase is measured by competition against ^{3}H-cAMP.

VI. Proteoglycan synthesis. Tissues or cells are incubated with $^{35}SO_4$ and then homogenized in 4 M guanidine hydrochloride to dissociate proteoglycans. After removing insoluble material by centrifugation, the supernatant solu-

tion is passed over a gel filtration column to remove free $^{35}SO_4$, the proteoglycans in the void volume fraction are collected and radioactivity measured to determine the extent of incorporation.

Cell lines used in these studies

Chick embryo fibroblasts: prepared by digesting frontal bones from 15 day chick embryos with crude collagenase and culturing the released cells. Subcultures in the second to fifth passage are used.

Chick embryo chondrocytes: prepared from sternum.

L-929--a line of mouse embryo fibroblasts established about 30 years ago by use of a chemical carcinogen.

BALB-3T3--a contact inhibited line of mouse embryo fibroblasts isolated by Todaro and Aaronson and subclones of this line, P3 and 714, which are more stringently contact-inhibited than the original culture, and P13, which has lost density dependence.

Ki-3T3-234--a line established by Aaronson by transformation of BALB 3T3 cells with Kirsten sarcoma virus. These cells do not produce viral particles unless super-infected with murine leukemia virus.

Mo-3T3--BALB 3T3 cells transformed with Moloney sarcoma virus.
SV-3T3--BALB 3T3 cells transformed with SV-40 virus.

Embryonic and adult human diploid fibroblasts and primary Syrian hamster embryo(SHE) fibroblasts were purchased commercially.

NQT-SHE--A cell line derived by 4-nitroquinoline-1-oxide transformation of SHE cells.

BALB 3T3 subclones 3 and 714 were transformed with a temperature sensitive mutant of Kirsten sarcoma virus (KSV) containing Moloney leukemia virus (MLV) helper to give a mass infected and transformed line, tsKi(MLV)-3T3. Control lines infected with only MLV were also produced (MLV-3T3). A non-producer subclone, tsKi-3T3-714 was derived from the transformed culture of 3T3-714.

Major Findings:

I. The Role of Ascorbic Acid in Collagen Metabolism

Our previous results showed that type I collagen synthesis was markedly decreased in parietal bone from scorbutic guinea pigs. This decrease was directly correlated with the extent of weight loss which occurred in the third and fourth weeks of scurvy, rather than with defective proline hydroxylation which occurred quite early in the disease. Essentially the same effects were observed in articular and costal cartilage which synthesize type II collagen. The fact that ascorbate is directly involved in

proline hydroxylation previously had led to the assumption that defective connective tissue metabolism in scurvy was mediated through this reaction.

We also found that decreased collagen synthesis could be observed in bone and cartilage of acutely fasted guinea pigs which were receiving ascorbate supplements, confirming that collagen synthesis could be regulated by other nutritional factors and suggesting that vitamin C deficiency indirectly led to a fasting state by inducing anorexia. Synthesis of proteoglycan, another extracellular matrix component, also was inhibited in cartilage of scorbutic and fasted guinea pig. In scurvy the decrease was coordinate with the effect on collagen. These effects were very specific since total protein was not significantly affected. During the past year, we have continued studies on the molecular mechanism for the scurvy-induced inhibition of collagen synthesis in bone and cartilage and on the mechanism for the inhibition of proteoglycan synthesis.

A. Mechanism of Inhibition of Proteolycan Synthesis.

Proteoglycan monomers consist of a core protein with a large number of glycosaminoglycan (GAG) chains attached through a serine-xylose linkage. Our current studies indicate that in scorbutic cartilage, core protein synthesis is unaffected, the size of proteoglycan monomers is slightly smaller than normal, but GAG chains are of normal length and contain the normal ratio of glucosamine and sulfate. Furthermore, studies in which β-xyloside was used as acceptor of GAG chains demonstrated that the defect in synthesis is beyond transfer of xylose to core protein. Therefore, we postulated that either the synthesis or the activity of the enzyme catalyzing the next sugar transfer, galactosyl transferase, may be decreased. In collaboration with Dr. Nancy Schwartz of the University of Chicago, this enzyme activity was measured in costal cartilage and was found to decrease coordinately with collagen and proteoglycan synthesis during the third and fourth weeks of scurvy, supporting our hypothesis.

B. Hormone Levels in Sera of Scorbutic and Acutely Fasted, Vitamin C Supplemented Guinea Pigs.

In order to obtain further evidence that regulation of collagen and proteoglycan is similar in acute scurvy and fasting, and to obtain some clues concerning changes in possible regulatory factors, serum levels of selected hormones known either to be altered in fasting or to effect collagen or proteoglycan synthesis in other systems, were measured in control and experimental animals. These hormones include cortisol, insulin and somatomedins. Preliminary results using a bioassay in which stimulation of 3T3 cell growth was measured by ^{3}H-thymidine incorporation indicated that there was a gradual reduction in serum stimulatory factors after two weeks of scurvy paralleling the decrease in collagen and proteoglycan synthesis. Essentially the same effect was seen using serum from fasted, vitamin C supplemented animals. The component lacking from scorbutic serum is not EGF and tentatively has been identified as a somatomedin.

C. Molecular Mechanism for Decreased Collagen Synthesis in Scurvy.

Total RNA was extracted from articular cartilage of guinea pigs at various times after placing them on an ascorbate-deficient diet. Dot-blot hybridization assays of type II (cartilage) mRNA with a type specific cDNA probe was carried out on samples from scorbutic and control animals. The results showed a dramatic decrease in type II collagen mRNA after the second week. This correlates with the period when collagen synthesis decreased and suggests that the major mode of regulation in scurvy is through alterations in mRNA concentrations.

II. Production of New Collagen Types by Chemically Transformed Cells

Transformation of SHE cells by nitroquinoline oxide produces a major alteration in the collagen phenotype. Initial experiments indicated that synthesis of type I procollagen, the major collagenous product of the parent cells, was greatly decreased or perhaps even completely suppressed in a transformant, NQT-SHE. This conclusion was based on analysis of secreted proteins by SDS/PAGE and by cell-free translation of RNA extracted from SHE and NQT-SHE cells. The major collagens produced are two unusual types unlike any previously described. They both appear to be homopolymers of approximately 125-130K molecular weight with no disulfide bonds. Both contain helical regions of 95K molecular weight resistant to mild pepsin digestion but cleaved by bacterial collagenase. One is precipitated by 33% saturated ammonium sulfate, like other collagen types, but differs in that its secretion is not dependent on hydroxylation of proline and thus does not require ascorbate for optimal secretion. The other molecule is unusual in that it is not precipitated at 33% saturated ammonium sulfate but is precipitated at 50% saturation. This allows separation of the two collagens by ammonium sulfate fractionation.

Analysis of cells pulse labeled with radioactive proline for 30 min, which is prior to the secretion of any collagens, revealed the presence of both collagens and the absence of pro α_1I chains. These results indicate that these unusual collagens are primary products of the cells and not produced by extracellular degradation of other collagen types. Recent results of V-8 protease peptide mapping suggest that there is some similarity between these polypeptides and the α_2I chain of type I collagen. Dot-blot hybridizations with a pro α_1I specific cDNA probe indicated that the absence of pro α_1I synthesis can be explained by the absence of mRNA coding for this chain.

Proposed Course of Research:

I. Role of Ascorbate in Connective Tissue Metabolism

We are continuing analysis of normal and scorbutic guinea pig serum to determine possible differences in hormone content. In addition, we are growing chick embryo chondrocytes in culture as a test system to determine whether scorbutic guinea pig serum will influence the level of type II collagen and proteoglycan synthesis and whether or not ascorbate plays a direct role in regulation. These cells are relatively easy to prepare and

exhibit high rates of collagen and proteoglycan synthesis. This approach was taken because of problems encountered in trying to establish long term cultures of guinea pig bone and cartilage and in isolating sufficient quantities of cells from guinea pig tissues.

II. Production of New Collagen Types by the Chemically Transformed Cells NQT-SHE

A. Further Characterization of the Collagenous Proteins (N-Collagens)

Both proteins will be purified by standard techniques used for procollagens. The procollagens will be separated by ammonium sulfate fractionation and chromatographed on DEAE-cellulose. Further fractionation techniques which can be applied if necessary are gel filtration and affinity chromatography on ligands such as the gelatin binding region of fibronectin. These procedures will also yield comparative information with respect to the behavior of other procollagens and collagens. The 95K helical region (collagen) can be purified easily by treating the 125-130K procollagen forms at mild pepsinization conditions. Further purification and characterization of these chains can be achieved by chromatography on CM-cellulose under denaturing conditions. Properties of the purified procollagen and collagen forms will be examined such as amino acid composition and additional peptide mapping. The purified proteins will be used to prepare antibodies for structural studies and immunocytochemical work.

B. Purification of Normal Syrian Hamster Type I Collagen

Type I collagen from Syrian hamster skin has been extracted and partially purified. The native molecule will be completely purified and it and the constituant α_1I and α_2I chains will be used to prepare antibodies. These will be useful in determining whether the N-collagens are related to α_2I chains.

C. Investigation of the Role of Proline Hydroxylation in Secretion of NQT-SHE Collagens

Preliminary results suggest that secretion of one of the two collagens is not dependent on hydroxylation of proline. Hydroxyproline stabilizes the collagen helix and thus at body temperature the Tm of hydroxylated procollagen is sufficiently high for the molecule to be almost entirely in the helical form, which appears to be required for secretion. The fact that secretion of this collagen does not require the presence of hydroxyproline, although there is a helical region, suggests that other means of stabilizing the helix may be utilized in this case. The Tm of purified hydroxylated and unhydroxylated forms will be determined by protease susceptibility.

Significance to Biomedical Research and the Program of the Institute:

Determining the precise mechanism by which vitamin C affects collagen synthesis should lead to a more accurate use of this vitamin. Our studies suggest that

there may be some functions for ascorbate previously not recognized. This information may be relevant to the suggested use of vitamin C as an anticancer agent.

The alteration of the collagen phenotype by transformation provides further information on biological changes caused by carcinogenic agents as well as useful models for studying cellular differentiation. In addition, the identification of the unusual collagens produced by a chemically transformed cell line may reveal additional functions for collagenous proteins.

Publications:

Majmudar, G., and Peterkofsky, B.: Cyclic AMP-independent processes mediate Kirsten sarcoma virus-induced changes in collagen production and other properties of cultured cells. J. Cell. Physiol. 122: 113-119, 1985.

Spanheimer, R.G., and Peterkofsky, B.: A specific decrease in collagen synthesis in acutely fasted, vitamin C-supplemented, guinea pigs. J. Biol. Chem. 260: 3955-3962, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05202-18 LB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Isolation, Fractionation, and Characterization of Native Nucleoproteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

O. Wesley McBride	Chief, Cellular Regulation Section	LB	NCI
R. Balachandran	Fogarty Associate	LB	NCI
W. Evans	Research Chemist	LB	NCI

COOPERATING UNITS *(if any)*: Drs. Steve Tronick & Stuart Aaronson, LCMB, NCI; Drs. E. Hildebrand & D. Nebert, DP, CH; Drs. H. Krokan & C. Harris, NCI; Dr. Stanley Korsmeyer, MET, NCI; Dr. John Minna, DCT, NCI; Dr. Ed Max LIG, NIAID; Dr. Benoit deCrombrugghe, LMB, NCI; Drs. D. Givol & M. Oren, Weizmann Institute, Israel.

LAB/BRANCH: Laboratory of Biochemistry, DCBD

SECTION: Cellular Regulation Section

INSTITUTE AND LOCATION: National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	2.0	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to develop methods for gene transfer to mammalian cells and to use these techniques for gene mapping, analysis of gene expression, and cloning eukaryotic genes. Many independent somatic cell hybrid lines segrating human chromosomes have been isolated and the human chromosome content of each line determined. Analysis of these lines with isotopically labeled cloned DNA probes has previously allowed assignment to specific human chromosomes, and sometimes regional localization, of human cellular onc genes, immunoglobulin genes and pseudogenes, α, β, and γ fibrinogen genes, members of the metallothionein multigene family, and calcitonin. Similar procedures have been used to localize other human genes including cytochrome P_1-450 to chromosome 15, L-myc to chromosome 1 p, follicular lymphoma breakpoint to chromosome 18, and a gene and pseudogene for J protein to chromosome 4 and 8, respectively. Other human genes which have been chromosomally and regionally mapped by both somatic cell hybrid and in situ chromosome hybridization techniques are p53 tumor antigen (chromosome 17p13), collagen type III (chromosome 2q11-13), and β polymerase (chromosome 8q24). In a second area of investigation, rearrangements of cellular protooncogenes have not been detected in guinea pig leukemia. Evalution of c-abl in guinea pig leukemia by cloning, chromosomal mapping, and expression is in progress.

Project Description

Objectives:

Development of methods for gene transfer to mammalian cells and use of these procedures for chromosomal and regional gene mapping, analysis of gene expression in normal and neoplastic cells, and cloning eukaryotic genes.

Methods Employed:

Tissue culture procedures, including plating in selective media, cloning, selection of cell mutants, and isolation of somatic cell hybrids after interspecific fusion of microcells with whole cells or cell/cell fusion. Metaphase chromosome isolation and purification by isopycnic and velocity sedimentation; gene transfer to mammalian cells by uptake of purified metaphase chromosomes, high molecular weight, eukaryotic DNA, or cloned recombinant DNA with subsequent isolation of biologically transformed colonies in selective media. Isolation and characterization of gene products by chromatography, electrophoresis, isoelectric focusing, and immunochemical procedures. Karyological analysis of hybrid cells and transformed cells by fluorescence, phase, and bright-field photomicroscopic techniques. Cloning of eukaryotic genes in prokayotic vectors and analysis of cloned genes by standard recombinant DNA techniques.

Major Findings:

I. Eukaryotic Gene Mapping by Analysis of Somatic Cell Hybrids

Analysis of somatic cell hybrids segregating human chromosomes permits the localization of human genes to specific chromosomes. Genes also can be regionally localized on a chromosome by analysis of hybrids containing well-characterized human chromosome translocations or spontaneous breaks. Several large series of independent human-rodent hybrid cell lines have been isolated in selective medium, and subcloned to obtain segregant hybrid cell populations containing a reduced, and relatively homogeneous, content of specific human chromosomes. The specific human chromosomes present in hybrid cell lines and subclones were determined by isoenzyme analyses and sometimes confirmed by karyotyping. DNA was simultaneously isolated from these same hybrid cell populations, and DNA fragments were transferred to nitrocellulose after restriction endonuclease digestions and agarose gel electrophoresis. Hybridization of the transferred DNA with isotopically labeled, cloned DNA probes thereby permitted assignments of genes to specific human chromosomes and regional chromosome localization in many cases. These techniques do not require expression of the relevant gene in hybrid cells, and allow genes, pseudogenes, and molecularly rearranged genes to be independently mapped to specific chromosomes. Previous collaborative studies with Phil Leder et al, Stuart Aaronson et al, and J. Kant et al have resulted in the human chromosomal mapping of heavy and light chain immunoglobulin genes and pseudogenes, eight proto-oncogenes, and the α, β, and γ fibrinogen genes, respectively. Human calcitonin and the metallothionein multigene family have also been mapped to 6 different chromosomes previously in collaboration with B.D. Nelkin et al. and Dean Hamer and C. Schmidt, respectively.

A. Subchromosomal Localization of the Human p53 Cellular Tumor Antigen Gene

The p53 tumor antigen is found in elevated levels in a large variety of transformed cells. It has many interesting similarities to the myc protooncogene family with respect to overall structure, sequence features, and property of binding to nuclear proteins. Givol and Oren recently cloned the full-length p53 from a human cDNA library and a 2.1 kb fragment containing the entire coding region was used to probe our hybrid cell DNAs. The p53 gene was mapped to human chromosome 17 and further localized to the most distal band on the short arm of this chromosome (17p13) by screening hybrids containing a well-characterized reciprocal translocation involving chromosome 17. We also confirmed these results by in situ hybridization of metaphase chromosome spreads with an ^{125}I-labeled p53 cDNA probe. The p53 location does not correspond to the chromosome 17 breakpoint (17q11) frequently reported in Acute Promyelocytic Leukemia.

B. Human L-myc Localization

Chromosomal rearrangements involving c-myc have been reported as consistent findings in Burkitt's lymphoma and mouse myeloma. It has recently become apparent that other cellular sequences with homology to c-myc are sometimes amplified or rearranged in other human tumors. Minna and colleagues have found rearrangements or amplification of c-myc, N-myc, mL-myc in two-thirds of small cell lung carcinomas. To determine the chromosomal location of L-myc, a cloned genomic fragment from the 5' end of the gene exhibiting little homology to other myc family members was used to probe our hybrid DNAs. In collaboration with J. Minna et al, this gene was mapped to human chromosome 1 distal to the N-ras locus on the short arm. Regional localization was obtained by analyzing hybrid DNAs containing human chromosome translocations. Subsequent in situ metaphase chromosome hybridizations by G. Hollis and I. Kirsch in Minna's lab have confirmed this location and narrowed the locus to band 1p32. Non-random chromosome aberrations involving this region have been previously reported in human tumors including neuroblastomas.

C. Chromosomal Localization of Human Cytochrome P-450 Genes

The membrane-bound multicomponent cytochrome P-450 system metabolizes endogenous and foreign substrates including almost all drugs, chemical carcinogens, and other environmental pollutants. Hence, this highly-conserved system is relevant to chemical carcinogenesis, drug toxicity, and other physiological and pathological processes. Full-length, human and mouse P-450 cDNAs have recently been cloned and sequenced by D. W. Nebert et al. Analysis of our hybrids with these cDNA probes, in collaboration with D. Nebert and E. Hildebrand, permitted assignment of the P_1-450 structural gene to human chromosome 15.

D. Chromosomal Breakpoint in Follicular Lymphoma

Non-random chromosome translocations involving the heavy chain Ig locus at 14q32 and specific sites on other chromosomes have been reported for

several types of human B-cell lymphomas and a t (14;18) (q32;q21) occurs in over 60% of follicular lymphomas. Using heavy chain Ig probes to detect unexpected IgH rearrangements, a sequence containing the putative breakpoint was cloned by S. Korsmeyer et al from a recombinant DNA library prepared from a follicular lymphoma. A probe prepared from the non-IgH region of the insert was used to screen our hybrid cell DNAs. This probe mapped to human chromosome 18 indicating that the chromosomal breakpoint has been cloned and that it may be possible to isolate a new transforming gene from these recombinants.

E. Mapping of a Diffuse Lymphoma Gene

S. Tronick et al have recently cloned a transforming sequence isolated from NIH 3T3 cells after transformation of the cells with DNA from a human Diffuse Lymphoma. This transforming sequence does not hybridize with known oncogenes even under reduced stringency. A probe prepared from one end of this cloned sequence maps to human chromosome 3 in our hybrid panel. Restriction mapping and Southern analyses of genomic blots by Tronick et al suggests that the cloned sequences probably contain a rearrangment with respect to both normal and lymphoma genomic DNA. Probes prepared from other portions of these cloned sequences are currently being used to screen our hybrid cell DNAs to determine their chromosomal location. The evidence currently suggests that donor chromosomal DNA rearrangements occurred during transfection. It is not yet clear whether the cloned sequences represent a transforming gene in the lymphoma or the transforming capacity arose from activation of a normal protooncogene by the rearrangement in culture.

F. Mapping of Human β-polymerase

S. Wilson and colleagues have recently cloned genes for α and β-DNA polymerases and a DNA binding protein from a rat c-DNA library in a λgt11 expression vector using immunological screening. Since these genes are obviously important in cell proliferation and potentially important in neoplasia, collaborative studies were taken to chromosomally map them in our hybrids using the heterologous rat probes under reduced strigency. Beta polymerase was unambiguously mapped to chromosome 8 and our preliminary results from in situ chromosome hybridization suggests that it is located on the distal portion of the long arm in the same region as the c-myc locus (8q24).

G. Human Collagen III Location

At least one class of inherited disease, clinically identified as the Ehlers Danlos type IV syndrome, has been found to be associated with an important decrease in the levels of type III collagen in skin, whereas levels of type I collagen are unaffected. Symptoms of this disease are also distinct from those of inherited diseases associated with defects in type I collagen. In collaboration with B. deCrombrugghe, a short fragment of human Col III cDNA (with no appreciable homology to other collagen types) was used to map the gene in hybrids, and it was localized to the long arm of human chromosome 2. Our recent in situ hybridization

studies indicate that this gene is probably located just below the centromere at 2q11-13.

H. Mapping of J Protein Gene and Pseudogene

The J protein interact with IgG molecules to form IgG pentamers. Ed Max et al., recently cloned the human J protein gene and we have collaborated in mapping it to human chromosome 4 whereas a J chain pseudogene is present on chromosome 8.

II. Identification of Transforming Genes in Guinea Pig Leukemia (GPL) and Human Chronic Myelogenous Leukemia (CML)

A Guinea Pig Leukemia model developed by W. Evans has many similarities to human CML. Identification of the transforming genes in these leukemias is required to firmly establish this relationship. Collaborative studies with W. Evans have been undertaken for the purpose of identifying and cloning these transforming genes.

A. Analysis of GPL DNA for Rearrangement of Cellular Proto-Oncogenes

High molecular weight DNA was isolated from guinea pig leukemia, and corresponding normal myeloid, cells representing both blast crisis and the differentiated phase of leukemia. The DNAs were examined by Southern analysis after complete digestion with various (i.e. Eco RI, Bam Hl, HinD III, Kpn I, Sac I, Xba I) restriction endonucleases using ^{32}P-labeled, nick translated viral onc gene probes. Hybridizing cellular sequences were detected with homology to 12 different viral onc genes, but no reproducible differences in the pattern of hybridizing fragments could be found between normal and leukemia cellular DNAs. Particular effort was devoted to detection of any c-abl rearrangement using probes representing the 5', 3', and middle portion of the v-abl sequence. These results indicate that rearrangements of cellular proto-oncogenes in GPL are not detectable by this method. However, these findings do not exclude such rearrangements, since c-abl rearrangements are also not detected in human chronic myelogenous leukemic DNAs by this method even though chromosomal rearrangements of c-abl are known to be present.

B. Analysis of Guinea Pig Leukemia Cells for Altered c-abl Protein

An altered (P210) c-abl protein with associated tyrosine protein kinase activity was reported in human CML cells by Konopka et al. Immunoprecipitates of ^{35}S-methionine labeled guinea pig leukemia cells have been examined for altered protein using v-abl antiserum kindly provided by Dr. O.W. Witte. Cross-reactivity was not adequate for reliable analysis and we are awaiting the availability of antiserum from commercial source in the future to repeat these assays.

C. Northern Analysis for Quantitative or Qualitative Alteration in c-abl Transcripts

Undegraded RNA preparations from normal and leukemic guinea pig cells

have been examined recently for altered transcripts by Northern analysis using v-abl probes but results are incomplete. Consistent alterations of c-abl transcript size has recently been reported in human CML DNA.

Proposed Course of Research:

Chromosomal assignment and regional localization of human genes, especially proto-oncogenes and genes involved in carcinogenesis or neoplastic transformation, will continue through analysis of our panel of somatic cell hybrids. Our gene mapping analyses now also routinely include chromosomal in situ hybridization using nick-translated probes labeled with ^{125}I-dCTP. Satisfactory, but suboptimal, results have been obtained over the past year using modifications of of the general procedure of M. Harper. We will explore other techniques to enhance sensitivity and reduce the non-specific background obtained. A human Alu repetitive sequence, metallothionein c-DNA sequence, and several different v-abl sequences have been cloned into plasmid vectors containing the SP6 and T7 promoters. Control experiments have confirmed the fact that fulllength riboprobes can easily be prepared from these vectors using SP6 or T7 polymerases. Biotinylated riboprobes will be prepared, and optimal conditions for the fluoresceinated avidin-biotin sandwich detection technique will be established using the Alu and metallothionein riboprobes as our initial standards for repetitive and unique sequence families, respectively. If the sensitivity is inadequate for the detection of unique sequences, modifications using the biotimylated peroxidase-avidin complex system will be examined. If none of the biotin-avidin systems has sufficient sensitivity for unique sequence detection, ^{3}H or ^{125}I-labeled defined riboprobes of several kb length will be used. Techniques for chromosomal banding using biotimylated riboprobes of moderately repeated sequences will also be developed and compared with conventional banding procedures such as trypsin-geimsa banding.

Riboprobes containing v-abl sequences will be used for in situ chromosome hybridization to map c-abl in the guinea pig genome and determine whether this gene is located at the deletion/translocation site on chromosome 1 in guinea pig leukemic cells. Leukemic and normal guinea pig genomic DNA and cDNA libraries will be prepared. They will be screened initially for abl sequences and later for other oncogenes by hybridization ribosomes. Alterations of onc genes in leukemic cells will be detected by hybridization of plaque transfers with comparison with their normal counterparts using heteroduplexing and restriction mapping as the initial criteria.

Transfection assays will be repeated using cotransfection with pNeo and high molecular weight guinea pig leukemic DNA using the NIH 3T3 and nude mouse assays systems. Possible development of an in vitro colony forming assay for guinea pig leukemic cells will be explored.

Publications:

Hildebrand, C.E., Gonzalez, F.J., McBride, O.W., and Nebert, D.W.: Assignment of the human 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible cytochrome P_1-450 gene to chromosome 15. Nucl. Acids Res. 13: 2009-2016, 1985.

Bakhshi, A., Jensen, J.P., Goldman, P., Wright, J.J., McBride, O.W., Epstein, A.L., and Korsmeyer, S.J.: Cloning the chromosomal breakpoint of t(14;18) bearing human lymphomas: Clustering around J_H on chromosome 14 and near a transcriptional unit on chromosome 18. Cell (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05203-17 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunochemical Purification and Characterization of Immunocytes and Components

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M.G. Mage	Immunochemist	LB	NCI
L.L. McHugh	Biologist	LB	NCI
L. Romani	Fogarty Visiting Fellow	LB	NCI
B. Nardelli	Fogarty Visiting Fellow	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our goal is the development of cell separation methods for the specific isolation of immune cells, particularly for varieties of antigen-reactive cells (ARC) involved in cellular immune reactions, and for their subcellular fractionation in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. Populations of cells containing ARC are tested for binding to the cell surface antigens of target cells attached to insoluble supports. Separated populations are tested for cytotoxic effector cells (CTL) and their precursors, for activity in allograft rejection and graft-versus host reaction and in the mixed lymphocyte reaction. T cell subpopulations from thymus and spleen are also separated by and characterized with specific reagents, such as peanut agglutinin and fluorescent antibodies to the Lyt and CTL differentiation antigens, by flow cytometry. Surface molecules of target cells are isolated to test their binding to ARC. Monoclonal antibodies are prepared against CTL and CTL-derived cell lines in order to characterize their surface antigens.

GPO 914-918

Project Description

Objectives:

The goal of this project is the development of methods for the specific isolation of immune cells, particularly for different types of antigen-reactive cells (ARC) involved in cellular immune reactions to alloantigens, to study the ontogeny of these cells, to study their roles, alone and in combination, in several *in vitro* and *in vivo* cellular immune reactions, and for their immunochemical characterization in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. In particular, we study the differentiation of cytotoxic effector cells (CTL) from precursors (CTLp) in thymus and spleen, the molecular reactions between immune cells and antigens bound to target cell surfaces, and the role of T cell subpopulations in *in vivo* cellular immune phenomona such as graft rejection and the graft-versus-host reaction.

Methods Employed:

ARC, obtained from normal mice or from mice immunized with allogeneic tumor cells or normal cells are incubated on surfaces coated with allogeneic or syngeneic target cells or on surfaces coated with antibodies to cell surface molecules. Graft-versus-host activity (GVH) of nonadherent cells or of released adherent cells is measured by the Simonsen spleen weight assay in neonatal F1 mice. CTL activity is measured by ^{51}Cr release from target cells. Stimulation by alloantigen is measured by the mixed lymphocyte reaction and by generation of CTL *in vitro*.

T cell subpopulations from thymus and spleen are separated and characterized by their reactions with specific reagents such as peanut agglutinin and antibodies to the LyT and CTL differentiation antigens. Cell surface molecules of target cells are isolated to test their binding to ARC. Monoclonal antibodies are prepared that react with CTL surface antigens.

Major Findings:

There has been progress in two areas in our studies on the role of separated lymphocyte subpopulations in immune reactions *in vivo*.

1. Continuing our collaborative study with Dr. John Kung, University of Texas, San Antonio, on the subsets of $Lyt2^+$ T cells distinguished by his rat monoclonal antibody "B4B2", we have studied the *in vivo* graft versus host (GVH) activity of the $B4B2^+$ and $B4B2^-$ lymphocytes and found that both subsets are active in this immune reaction.

2. In a collaborative study with Dr. P. Puccetti and colleagues of the University of Perugia, it was found that *in vitro* purified immune $Lyt2^+$ T cells had *in vivo* antitumor activity against intracerebral tumors in mice.

"Xenogenization" (i.e., induced mutation in tumor cells to increase their immunogenicity) is a potential approach to cancer immunotherapy that deserves further

exploration, in as much as many tumors fail to elicit an effective immune response. In a study on the immune response to such "xenogenized" tumor cells, we have been able to obtain a humoral response to a chemically xenogenized murine tumor cell line, previously identified only through its ability to elicit a cellular immune response.

Significance to Cancer Research:

The T cells that undergo maturation in the thymus (i.e. cytotoxic T cell precursors, and helper and suppressor T cells) are thought to be directly involved in anti-tumor immunity by means of cell-mediated tumor rejection and its augmentation and suppression. Knowledge of the antigen-binding characteristics, differentiation antigens, and reactivities of immune cells at different stages of differentiation may help in developing specific purifications of various types of immune lymphocytes reactive against tumor antigens and in specific suppression of the graft-versus-host reaction, which is a serious iatrogenic complication of cancer therapy with bone marrow transplantation.

Proposed Course of Research:

We plan to (1) continue to develop the general separation methodology based on specific binding of Ig-coated cells to anti-Ig-coated surfaces, (2) develop further separations of T cell subtypes, particulary CTLs, their precursors, helpers, and suppressors, and study their differentiation, (3) continue to study the contributions of these separated cell populations in vivo to immune reactions such as graft rejection, GVH reaction, and tumor rejection, (4) study the macromolecules involved in the binding of CTL to target cells, and (5) study the molecular requirements for immunogenic antigen presentation to CTLp.

In particular, we plan to continue to study the molecular requirements for immunization of CTL precursors, to continue the characterization of these cells with monoclonal antibodies, and to study methods for enhancing the susceptability of tumor cells to cellular immune reactions.

Publications:

Mage, M.: Separation of lymphocytes on antibody-coated plates. Methods In Enzymology. 108: 108-118, 1984.

Mage, M.: Cell separation on cellular monolayers. Methods In Enzymology 108: 118-125, 1984.

Romani, L., Nardelli, B., Bianchi, R., Puccetti, P., Mage, M., and Fioretti, M.C.: Adoptive immunotherapy of intracerebral murine lymphomas: Role of different lymphoid populations. Int. J. Cancer (in press).

Romani, L., Puccetti, P., Fioretti, M.S., and Mage, M.: Humoral response against murine lymphoma cells xenogenized by drug treatment in vivo. Int. J. Cancer (in press).

Romani, L., and Mage, M.: Search for class II molecular involvement in the response of $Lyt2^+$ cytotoxic T lymphocyte precursors to alloantigen. Eur. J. Immun. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05214-14 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA Synthesis in Mammalian Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

S. H. Wilson	Medical Officer	LB NCI
F. Cobianchi	Guest Worker	LB NCI
P. Kumar	Visiting Fellow	LB NCI
D. SenGupta	Visiting Fellow	LB NCI
B. Zmudzka	Visiting Associate	LB NCI

COOPERATING UNITS *(if any)*

J. Mitchell, NCI; J. Minna, NCI; A. Matsukage, Aichi Cancer Center; E. Baril, Worcester Foundation for Experimental Biology; S. Planck, U. of Oregon; P. Hoffee, University of Pittsburg, G. Wright, University of Massachusetts

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	5	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We characterized a set of newborn rat brain cDNAs to the mammalian DNA replication proteins currently under study in our group. These proteins are α-polymerase, β-polymerase and the single-stranded nucleic acid binding protein termed helix destabilizing protein-1. Six cDNAs for the binding protein were isolated and the nucleotide sequences of each was determined. One of these cDNAs selected for detailed study was a full-length copy of mRNA encoding the predominent species of the binding protein family in rodent tissue. This polypeptide has a molecular weight of 34,215 and is routinely purified as a truncated polypeptide of ~25,000-M_r. Our cDNA for rat β-polymerase was sequenced. This DNA appeared to be a full-length mRNA copy also, in this case encoding the 40KDa enzyme. Northern blot analysis of newborn rat brain polyA$^+$ RNA using this nick translated cDNA as probe revealed one predominant hybridizing RNA species of about the same dimension as the insert itself. Southern analysis of rat genomic DNA was consistent with a single copy gene. Similar results were obtained with human genomic DNA and the gene was localized to chromosome 8 using hybrid cell chromosome segregation techniques. β-polymerase levels in a number of human cell lines were determined and were found to be modestly elevated (2 to 3-fold) in lung cancer cells and in cells from patients with ataxia telangectasia. Using our cDNA for rat α-polymerase, Northern blot analysis of newborn rat brain polyA$^+$ RNA revealed a single hybridizing species of mRNA of about 4600 bases. This mRNA was 4-times longer than the cDNA itself and is long enough to encode the 1800 to 190KDa α-polymerase catalytic subunit described earlier. Southern blot analysis of rat genomic DNA was consistent with a single copy gene. In an attempt to obtain rat cell lines with amplified α-polymerase genes, we isolated cell lines resistant to the competitive inhibition of α-polymerase butylphenyldeoxyguanosine. Two of these cell lines were found to have increases in the amount of both α-polymerase enzyme activity and α-polymerase catalytic subunit as determined by Western blot analysis. Finally, experiments toward developing a system for study of polyoma virus DNA replication in vitro were continued.

Project Description

Objective:

The ultimate objective of this research program is to understand mechanisms of DNA synthesis in mammalian cells. Our main approach is the examination of DNA replication in vitro using purified DNA polymerases and other required proteins.

It is anticipated that these studies on the properties and specificities of the DNA replication proteins will, in conjunction with results from other workers, help answer important questions about mechanisms of DNA replication in the cell.

Methods Employed:

Procedures for assay and purification of DNA polymerases and other DNA proteins from mouse and other tissues are being used. These procedures involve subcellular fractionation, various types of ionic separation, gel filtration, immunobinding, HPLC, and affinity chromatography, and assays for DNA polymerases using a variety of methods. Cell fractionation procedures and characterizations of reaction products are performed using conventional techniques of rate-zonal centrifugation, scintillation spectrophotometry, thin-layer chromatography, and gel electrophoresis. Recombinant DNA technology is being used for preparation of cDNA libraries and expression vector clones containing gene sequences of DNA enzymes.

Major Findings:

I. Structure-Function Relationships of E. coli DNA Polymerase I Large Fragment (Pol I l.f.)

A. Termination Mechanism

Generally, Pol I l.f. forms DNA in a processive fashion, remaining associated with the template:nascent chain complex during many individual dNMP additions. In an earlier report, however, we described how the enzyme can be forced to terminate synthesis and dissociate from the template after only limited dNMP additions in synthetic homopolymer template-primer systems. We now have studied termination as a function of each nucleotide addition using a ϕX174 template-primer system. The results indicate that regions of single-stranded DNA which are secondary structure-free nonetheless contain strong "termination signals" for this enzyme. Synthesis is highly processive until a termination signal is encountered, at which point the chance for termination is increased. Comparisons of nucleotide sequence at several strong termination sites did not reveal common sequence features. On the other hand, this analysis showed that termination was much higher at each position where the incoming nucleotide was a pyrimidine.

II. Structure-Function Relationships of Mammalian α-Polymerases

DNA polymerase α heterogeneity in mammalian systems is well recognized, yet the functional and biochemical reasons for this property are largely obscure.

Our current approach toward study of this general problem is 1) characterization of α-polymerase genes and 2) analysis of proteins in crude extracts recognized by antibodies to α-polymerase.

A. Native Species of α-polymerase Identified by Antibody Probing of Western Blots

We prepared a new rabbit antiserum to purified Hela cell α-polymerase catalytic subunit and used this antiserum in Western blotting experiments with the crude homogenate of Hela cells. The results indicated that the native species of the catalytic subunit can be rapidly degraded during purification. This in vitro proteolysis results in complete loss of the native species and accumulation of lower M_r proteins that appear to represent limit digestion products. These findings, along with a modified purification involving various protease inhibitors, have identified the true native form of the catalytic subunit in Hela cells as a protein of apparent M_r ~ 180,000.

B. Identification of an α-polymerase cDNA Clone

In last year's report, we described antibody screening of a newborn rat brain cDNA library in λgt11. During the past year a positive phage was obtained, using the new polyclonal antibody described above that was raised against the purified 180 KDa catalytic subunit of Hela cell α-polymerase. This antibody is a patent inhibitor of α-polymerase activity in vitro and is highly specific such that it reacts only with the 180 KDa α-polymerase catalytic polypeptide in Western blots of crude unfractionated homogenates of Hela cells. Our positive phage was plaque purified, and the 1.2 kb insert was cloned into pUC9. Hybrid selection experiments indicated that this cDNA is complementary to α-polymerase mRNA, although we consider that confirmation of this cDNA is only preliminary at the present time.

Northern blot analysis of newborn rat brain polyA$^+$ RNA using the nick-translated cDNA as probe indicated a single predominant hybridizing mRNA species. The dimension of this mRNA was 4,600 bases, which is an appropriate size for encoding a 180 KDa polypeptide. Southern blot analysis of rat genomic DNA was consistant with a single copy gene. Further studies of this cDNA are in progress.

C. Attempts to Isolate Rat Cell Lines with Amplified α-polymerase Genes

In collaboration with P. Hoffee and G. Wright, we have isolated a panel of rat hepatoma cell lines that are resistant to the α-polymerase inhibitor butylphenyldeoxyguanosine. This inhibitor competes with dNTP binding sites on the enzyme. It was expected that resistant cell lines could be nucleotide pool size mutants or permeability mutants, as well as various types α-polymerase mutants. Levels of α-polymerase enzyme activity were found to be ~ 5-fold higher in two of the resistant cell lines than in the parent cells. These drug resistant cell lines also were found to contain higher amounts of α-polymerase catalytic subunit

as revealed by Western blotting experiments. These findings are still in the preliminary stage and will be reported in more detail in a later report.

III. Studies of β-polymerase cDNA

A. Characterization

In last year's report we described the isolation of a putative β-polymerase cDNA clone in λgtll. This clone was isolated with a polyclonal antibody to chick β-polymerase using a library from newborn rat brain poly A^+ RNA. The use of heterologous antibody and library was reasonable since earlier work had shown extensive amino acid sequence homology between β-polymerases purified from rat and chick. The cDNA insert in the positive λ-gtll clone was 1150 bp. Northern blot analysis of poly A^+ RNA from newborn rat brain, calf thymus, and human fibroblast cells indicated a predominant hybridizing RNA species of about the same dimension as the insert itself. The insert was subcloned into pUC9 and then into M13 for sequence analysis.

Purified rat and chick β-polymerase were subjected to trypsin cleavage and resulting fragments from each enzyme were used for amino acid sequence analysis. Partial amino acid sequence thus obtained was compared with the sequence deduced from an open reading frame in the nucleotide sequence of the cDNA insert. A perfect match of involving 26 amino acid residues was found, including two complete oligopeptides 6 and 9 residues long, respectively. This provided unambiguous evidence for the identity of the cDNA insert. Sequence analysis of the insert further revealed a poly A sequence at the 3' terminus and a 45 nucleotide 5' region preceding the first AUG codon. Southern analysis of rodent genomic DNA was consistent with a low copy number gene.

B. Chromosome Mapping

In collaboration with O.W. McBride, human genomic DNA was subjected to Southern blot analysis. The results indicated a low copy number for the β-polymerase gene and were consistent with a single copy gene. Further, it seemed clear that chromosomal localization was feasible using human-rodent somatic cell hybrids since restriction fragments containing the gene were not identical for human and rodent DNA. Southern blot analysis of DNA from a panel of human-rodent hybrids indicated that the β-polymerase gene is located on chromosome 8. Experiments using *in situ* hybridization techniques confirmed this localization.

C. Survey of Expression and Gene Arrangement in Human Lung Cancer Cells

Using Northern and Southern blot analysis in collaboration with J. Minna we found that a panel of cultured lung cancer cells were identical to normal human cells in expression of β-polymerase mRNA and in gene copy number and apparent arrangement in the genome. Further analysis of human cancer cells and cells from patients with ataxia telangectasia and xeroderma pigmentosum are underway.

IV. Studies of DNA Polymease β in Cultured Cells with Abnormal DNA Repair

A. Cells from Patients with Ataxia Telangectasia and Various Types of Lung Cancer

DNA repair is generally considered a key factor in determining the shape of the classical radiation survival curve of a cell line. In collaboration with J. Mitchell, we observed that radiation survival curves of certain abnormal human cells lines differed from the curves obtained with normal human fibroblasts. Therefore, we examined the level of the DNA repair DNA polymerase (β-polymerase) in the abnormal cells. These cells included cultured skin fibroblast lines from patients with ataxia telangectasia and lines from lung cancers of different histology. In both of these types of abnormal cells, the level of β-polymerase was higher than in normal skin fibroblast. The significance of these findings is not clear, but deficiency in this DNA polymerase was not associated with differences in radiation survival curves noted.

V. Studies of Helix Destabilizing Protein-1 cDNA Clones

Our cDNA library of newborn rat brain poly A^+ RNA in λgt11 was screened with a synthetic oligonucleotide probe corresponding to a five amino acid sequence in the N-terminal region of the calf $_{ss}$DNA binding protein, UP1. Six positive phage clones were isolated and each was plaque purified. The inserts were subcloned in pUC9 and then in M13 for eventual sequence analysis. One cDNA insert was selected for detailed study. This cDNA was 1706 bp long. Northern blot analysis of newborn rat brain poly A^+ RNA using the nick translated insert as probe revealed one predominant hybridizing RNA species of about the same dimension as the insert itself. The insert contained a poly A sequence at the 3' terminus and a single open reading frame starting 28 bases from the 5' terminus and extending 988 bases. This open reading frame predicts a 34, 215 dalton protein of 320 a.a. Residues 2 through 196 of this predicted sequence correspond precisely with the 195 residue sequence of calf UP1. This verified the authenticity of the cDNA insert and indicates strong sequence conservation between rat and calf for the first 196 amino acids of the binding protein. The 3' noncoding region contained 718 bp including an AATAAA signal 21 bp from the poly A sequence. Primer extension analysis showed that the cDNA was full-length except for 31 residues missing from the 5' end. Southern analysis of rat genomic DNA was consistent with a multigene family.

A cDNA-derived sequence of 124 amino acids in the C-terminal portion of the protein is not present in purified calf UP1. This sequence is 40% rich in glycine residues, and it is cleaved from UP1 during purification. This was evident from Western blotting experiments with the crude homogenate of mouse myeloma solid tumors. The results indicated that the native species of HDP-1 can be rapidly degraded duirng purification. This in vitro proteolysis results in complete loss of the native species and accumulation of lower M_r proteins that appear to be limit digestion products. These findings, taken together, have identified the true native form of the helix destabilizing protein in rodent tissues as a species of M_r=34,215.

VI. System for Study of DNA Replication In Vitro

For some years during detailed studies of purified mammalian DNA enzymes, we anticipated eventual use of these proteins in in vitro system closely reflecting in vivo replication. Based upon impressive in vitro replication results from several groups involving the E. coli origin of replication cloned into plasmid DNA, we were encouraged to attempt analogous experiments with the polyoma virus origin of replication cloned into the plasmid pML2. This plasmid (provided by J. Hassel) contains a 251 bp segment of polyoma virus DNA, can be readily prepared in mg quantities, and replicates in polyoma transformed mouse cells. We found that incubation of superhelical plasmid DNA in a replication mixture with crude extract from polyoma virus infected cells resulted only in a low level of dNMP incorporation due to nicking of the plasmid DNA and subsequent repair-like DNA synthesis. However, addition of a nuclear extract from Hela cells, as suggested by the recent work of Ariga and Sugano, increased dNMP incorporation; in this type of incubation about 70% of the DNA product represented extensive replication of plasmid DNA, was not covalently attached to the original plasmid DNA, and was semiconservatively formed. Some fully replicated plasmid molecules were observed as open circular and linear molecules, but no supercoiled Form I molecules were detected. Formation of this product DNA was not observed with a plasmid lacking the polyoma virus replication origin. Overall, the results of this work are still preliminary and will be described in more detail in a later report.

Significance to Cancer Research:

Detailed knowledge of the mechanisms of DNA synthesis is vital to our understanding the molecular biology of neoplasia. Many approaches are being used to investigate DNA synthesis in normal and neoplastic cells, and advances on all levels will prove useful in preventing, treating, and ultimately controlling cancer. Activity of DNA synthesizing proteins is necessary for maintenance of a rapid rate of cell division, and in some cases there is evidence that these proteins may play an important role in the development of the neoplastic state.

Proposed Course of Research:

To investigate replication activity of various DNA replication proteins in vitro using as template plasmid DNA containing the polyoma virus origin of replication.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05231-11 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Subunit Interactions in Enzyme Chemistry and Cellular Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

C.B. Klee	Chief, Macromolecular Interactions Section, LB, NCI		
M.J. Hubbard	Visiting Fellow	LB	NCI
J.L. Foster	Guest Research	LB	NCI
D.L. Newton	Research Chemist	LB	NCI
M.H. Krinks	Chemist	LB	NCI
J.R. Miller	Technician	LB	NCI
T. Jean	Visiting Fellow	LB	NCI
G.F. Draetta	Visiting Fellow	LB	NCI

COOPERATING UNITS *(if any)*

J. Schiloach, NIAMMD; Dr. P. Cohen, University of Dundee, Scotland; Dr. L. Heppel, Cornell University, Ithaca, NY; Dr. T. Burke and Dr. K. Rice, NIAMMD; J. Haiech CNRS, Montpellier, France; Dr. P. deCamilli, University of Milan, Milan, Italy

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Macromolecular Interactions Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.0	6.0	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Calmodulin, a ubiquitous calcium-binding protein recognizes changes in intracellular calcium concentration induced by external stimuli and transmits this information to cellular control are cAMP phosphodiesterase, protein kinases and a protein phosphatase (calcineurin) which confer upon calmodulin the ability to effect a tight couplin between cAMP and calcium. our major goal is to understand the mechanism of regulation of cellular processes by calmodulin. Previous work showed that binding of calcium to calmodulin is an order process that generates at least three different calmodulin conformers. Studies with calmodulin fragments and with a covalent adduct of calmodulin and and phenothiazine (CAPP Calmodulin) demonstrated that interaction of calmodulin with its several targets requires the integrity of different portions of the calmodulin molecule. Anticalmodulin drugs, such as phenothiazines, interact with either one of two sites located between residues 31 and 74 and 148. Calmodulin also contains two protein-binding sites located on each half of the molecule. Some enzymes need to interact at both sites to be activated. Others require only the one located in the COOH-terminal. With yet a third class of enzymes either one of the two can substitute for the native protein.

Project Description

Objectives:

To study the functional roles of protein subunits and protein-protein interactions. The system under investigation is the Ca^{2+}-dependent regulation of enzymes mediated by calmodulin. Emphasis is on cyclic nucleotide phosphodiesterase, and the Ca^{2+}-dependent protein phosphatase, calcineurin. These studies are undertaken to elucidate the roles of the two second messengers, Ca^{2+} and cAMP, in the regulation of cell function.

Methods Employed:

The project involves the study of properties of proteins, purified and analyzed by the usual techniques of protein isolation, including many types of chromatography and electrophoresis. The enzymes are studied by examination of their optical, hydrodynamic, kinetic, and immunochemical properties as well as by suitable chemical measurements and modifications. The roles of these proteins in cellular regulation are studied in cultured cell lines.

Calmodulin and Calcium Regulation of Cellular Activity:

The Ca^{2+}-dependent regulation of several cellular processes is mediated by the ubiquitous, intracellular Ca^{2+}-binding protein, calmodulin. Interactions of calmodulin with the Ca^{2+}-stimulated protein phosphatase, calcineurin and with the calmodulin antagonists, phenothiazines, are studied as model systems to elucidate the mechanism of action of calmodulin. Methods developed for these systems are applied to the study of interaction of calmodulin with other enzymes in collaboration with other investigators.

A. Mechanism of Action of Calmodulin

Stimulus-response coupling mediated by Ca^{2+} involves several successive steps: (1) transitory increase in intracellular Ca^{2+} from 10^{-7} to 10^{-6} - 10^{-5}M, (2) interaction of Ca^{2+} with the Ca^{2+} receptor, calmodulin (3) interaction of the activated calmodulin·Ca^{2+} complex with the various target proteins and (4) coordinated activation of several enzymatic reactions. We previously reported that Ca^{2+} binding to calmodulin occurs in a stepwise fashion. Large peptide fragments of calmodulin, generated by limited proteolysis with trypsin in the presence of calcium, encompass the amino (residues 1-77) and the carboxyl (residues 78-148) terminal halves of calmodulin. These fragments preserve much of the structure which they have in the parent molecule, bind calcium like calmodulin, and undergo similar calcium-dependent conformational transitions. Both fragments interact with the phenothiazines, in a calcium dependent manner. The effects of these fragments on calmodulin-activated enzymes are as follows: As shown by others (1) both peptides activate phosphorylase kinase (Kuznicki et al., 1981), (2) fragment 78-148 but not fragment 1-77 activates the (Ca^{2+}-Mg^{2+}) ATPase of erythrocytes (Krebs et al., 1984), (3) we found that neither fragment activates cyclic nucleotide phoshodiesterase or calcineurin (D. Newton). In spite of these differences in behavior, these fragments all interact with many of these enzymes when

coupled to a solid matrix. Phosphodiesterase, cAMP-dependent protein kinase, fodrin, and calcineurin are retained on a column of fragment 78-148 coupled to Sepharose in the presence of calcium and are eluted with EGTA-containing buffers. Calmodulin regulated protein kinase(s) is retained on the same column, but only at low ionic strength. Fragment 1-77 binds only phosphodiesterase and cAMP-dependent protein kinase. Thus, the enzymes have differing requirements for interaction with or activation by calmodulin. The enzymes and anti-calmodulin drugs have different modes of interaction with calmodulin. These observations have been used to develop selective affinity chromatographic procedures to purify calmodulin-binding proteins (W.C. Ni and G. Draetta).

The interaction site(s) of calmodulin with phenothiazines has been identified using a chemically reactive derivative, norchlorpromazine isothiocyanate synthesized for us by K. Rice and T. Burke (NIAMDD). A one-to-one, covalent phenothiazine-calmodulin adduct, CAPP-calmodulin, is formed in the presence of calcium but not in its absence. The attachment site is at Lys 75. UV-irradiation of CAPP-calmodulin followed by peptide mapping of the irradiated protein revealed the selective loss of tryptic peptides 31-74 and 107-148 suggesting that these two peptides contain phenothiazine interacting domains. CAPP-calmodulin interacts, with high affinity, with all calmodulin-regulated enzymes that have been tested. However, it exerts different effects depending upon the enzyme under study. It acts as a competitive inhibitor of calmodulin activation of phosphodiesterase and myosin kinase. It is a partial activator of calcineurin and a full activator of multifunctional calmodulin-dependent kinase, phosphorylase kinase and (Ca^{2+}-Mg^{2+})ATPase. CAPP-calmodulin has no effect on the calmodulin-independent, calcium stimulated protein kinase called C-kinase. Thus, occupancy of a single phenothiazine-binding site on calmodulin does not prevent its interaction with its target proteins but modifies its ability to activate these enzymes. By attaching phenothiazines to calmodulin, we have transformed a low affinity ($K_i=10^{-6}M$) and non-specific calmodulin antagonist, chlorpromazine, into a high affinity ($K_i=10^{-9}M$) and specific antagonist for some but not all calmodulin-regulated enzymes. CAPP-calmodulin is presently being tested as a probe of the biological roles of calmodulin (D. Newton).

B. Regulation of Calcineurin

Calcineurin, a calmodulin stimulated protein phosphatase, is composed of two subunits: The large subunit, calcineurin A, is the catalytic component. Calcineurin A interacts in a calcium-independent fashion with the small subunit, calcineurin B. The latter is a calcium-binding protein, homologous to calmodulin. In collaboration with P. Cohen, we determined the sequence of calcineurin B and showed it to have a myristylated amino-terminus. This hydrophobic substituent could serve to anchor the protein in the membrane or to provide an interaction site for other proteins.

Calcineurin is partitioned among the soluble, membrane and cytoskeletal compartments of the cell. Brain calcineurin A and B were quantitated in homogenates after SDS gel electrophoresis. Calcineurin A was measured by

125-I-calmodulin binding. Contents of calcineurin A and B were determined by quantitative Western blot analysis of the gels treated with antibodies to calcineurin. Brain contains high levels of calcineurin (600-800 mg/kg) that are efficiently solubilized only in the presence of detergents such as CHAPS. Skeletal, cardiac and smooth muscle, liver, kidney, spleen, thymus and lung showed 1/10th to 1/20th as much calcineurin B as brain. The antibody to the bovine brain protein also detected calcineurin B in rat and rabbit tissues and Drosophila embryos. The antibody cross-reacted poorly with calcineurin A in tissues other than brain. However, anticalcineurin immunoglobulins coupled to Sepharose selectively adsorbed the Ca^{2+}-dependent, phenothiazine-inhibited, protein phosphatase. Polypeptides eluted from the antibody-Sepharose had M_r similar to those of calcineurin A and B. Thus, calcineurin has a wide distribution and has a similar subunit composition in different tissues. Whereas the Ca^{2+}-binding subunits (calcineurin B) are antigenically related, the catalytic units (calcineurin A) exist as different tissue specific isozymes (M. Krinks).

The catalytic subunit of calcineurin, calcineurin A, interacts with calmodulin in a calcium dependent manner and this interaction is prevented by limited proteolysis of calcineurin which converts calcineurin A to a smaller product without affecting calcineurin B or interaction of calcineurin A with calcineurin B. The interaction of calmodulin with calcineurin is weakened by acetylation of a single lysyl residue of calmodulin. Conversely, the reactivity of lysyl residues of calmodulin is affected by the calcium-dependent binding to calcineurin. Lys 94 is not involved in the interaction between the two proteins whereas Lys 148, 75, 77 and one or more lysines in the NH_2 terminal fragment are affected by the interaction between the two proteins. These experiments suggest a complex, multisite, interaction which may account for the observed high affinity of calmodulin for calcineurin (A. Manalan).

C. Identification of Other Calcium Regulated Proteins

Most eukaryotic cells contain several Ca^{2+}-binding proteins, which are structurally related to calmodulin, but are tissue and species specific. These proteins cannot usually substitute for calmodulin but may act as modulators of the calcium response.

A brain specific calcium-binding protein (CBP-18) was purified by A. Manalan from the bovine. This protein, like calcineurin B, does not activate calmodulin-regulated enzymes, and interacts with a calmodulin binding protein which is being purified by combined affinity chromatography on calmodulin-Sepharose and anti-CBP-18 coupled to Sepharose.

In collaboration with Dr. L. Heppel we purified oncomodulin from Morris rat hepatoma according to the procedure of Durkin et al. (1983). Oncomodulin, is a calcium-binding protein found only in tumor cells. After HPLC purification, the preparation, in general, had the properties and amino acid composition of the material described by these authors. However, we were unable to confirm the reported stimulation of cyclic nucleotide phosphodiesterase and found that the protein also failed to activate calmodulin regulated enzymes, protein kinases or calcineurin.

Significance to Biomedical Research and the Program of the Institute:

The proteins being studied are important enzymes in the control of cell metabolism and are regulated by protein-protein interactions. cAMP phosphodiesterase is one of the two enzymes responsible for the control of cAMP levels. Calcineurin, as a Ca^{2+} regulated protein phosphatase can modulate the function of many enzymes and other proteins whose activity is regulated by phosphorylation. cAMP and Ca^{2+} mediated phosphorylations are critical for the regulation of cell growth and differentiation. Calmodulin, as a common modulator of these, and many other enzymes, provides a link between cyclic nucleotide levels and Ca^{2+} regulation of cellular processes. The ability of calmodulin to regulate a large number of biological processes represents a novel mechanism with great potential physiological importance. The N-terminal myristylation of calcineurin and of the catalytic subunit of cAMP dependent protein kinase (Carr et al., Proc. Natl. Acad. Sci., 79, 1982, 6128), two important enzymes in cellular regulation may prove to be useful in understanding the role of this postranslational modification in cellular transformation. Myristyl residues, identified at the NH_2 terminus of p60SRC, the gag polyproteins and of gag - onc fusion proteins in mammalian transforming retroviruses may direct the membrane association of these proteins (Henderson et al., Proc. Natl. Acad. Sci., 80, 1983, 339; Pellman et al., Nature, 314, 1985, 374).

Future Course of Research:

We will continue to study the structure of calmodulin in solution in order to characterize the Ca^{2+} conformers responsible for multiple functions, and to identify the calmodulin interacting site(s) with its target proteins. Our efforts will be concentrated on the study of the structure-function relationships of the calmodulin-regulated protein phosphatase calcineurin, and on the elucidation of the biological role of this protein. We will try to clarify the linkage between the two second messengers, Ca^{2+} and cAMP, by studying the effects of calmodulin and Ca^{2+} on cAMP-dependent protein kinase and protein phosphatase. These studies will be carried out at the molecular level with purified and characterized proteins and at the cellular level with cells in tissue culture to correlate the in vitro observations with physiological events. Among the model systems in which the role of calmodulin and other Ca^{2+}-binding proteins will be investigated are: the differentiation of neuroblastoma glioma hybrid NG-108-15 and in vitro transformation of cells. John Foster, who joined the laboratory last summer, is attempting to clone and characterize the calcineurin gene in Drosophila and hopes to obtain mutants of this protein to study the behavioral effects of these mutations.

Publications:

Manalan, A.S., and Klee, C.B.: Calcineurin, A calmodulin-stimulated protein phosphatase. Calcium in biological systems. In Rubin, R. (Ed.): Vol I: Metabolic and Functional Aspects of Calcium Action. London, Plenum Press, 1985, pp. 307-315.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05234-11 LB

PERIOD COVERED
October 1, 1984, to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Interrelations Between the Genomes of SV40 and African Green Monkeys

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Maxine F. Singer Chief, Nucleic Acid Enzymology Section LB NCI

COOPERATING UNITS *(if any)*

Professor R. Tjian, Department of Biochemistry, Univ. of California, Berkeley;
J. Saffer, Jackson Laboratories, Bar Harbor, ME

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Nucleic Acid Enzymology

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors B
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A cloned segment (450 bp) of the African green monkey (Cercopithecus aethiops) genome that contains DNA sequences homologous to the control region of simian virus 40 is being studied. Cellular transcripts that go in both possible directions from within the SV40-like region, which is hypersensitive to DNase I in monkey chromatin, have been detected. Also, the sequence provides information for initiation of transcription from vectors constructed by molecular cloning. The SV40-like region also is a bidirectional transcriptional start site in in vitro reactions using fractionated cell free extracts. In vitro transcription depends on the presence of a transcription factor (Sp1) that is also required for in vitro transcription from SV40 DNA itself but not for other host cell promoters tested. The Sp1 factor binds to the cloned monkey genomic region, as demonstrated by footprinting experiments using DNase I. The SV40 control region and the monkey segment compete for binding to Sp1. With both DNAs, the binding is to homologous regions containing multiple repeats of the sequence 5'-GGGCGGPuPu. The data suggest that there is a special class of cellular promoters that, like the SV40 promoter, depend on the presence of the short G-rich DNA segment and interaction with a specific factor, Sp1.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

A range of interactions occur between permissive cells and infecting viruses. Using simian virus 40 (SV40) as a model, we studied one aspect of viral-host interaction, namely, the use of common regulatory components. When SV40 enters a permissive monkey cell it uses the host machinery for the initiation of early viral transcription and the synthesis of early viral proteins. Subsequently, interaction of an early viral protein (T-antigen) with the viral genome is required for viral DNA replication and the synthesis of late proteins. These events suggested that the viral and host DNAs might share similar sequences that are recognized by a variety of enzymes and regulatory proteins. Earlier we searched for, found, and characterized DNA sequences in the monkey genome that are homologous to the control region of the SV40 genome. These regions, about 80 per genome and a few hundred base pairs in length, include sequences like those around the SV40 origin of DNA replication. In particular the homologous regions include multiple copies of a G·C rich repeat, 5'-GGGCGGPuPu, that is required for both early and late viral transcription; the influence of this sequence is thus bidirectional. We investigated the ability of one of the homologous monkey DNA segments to serve as a promoter for transcription of RNA. We found that the SV40-like region is hypersensitive to DNase I in monkey chromatin and serves to promote transcription in both possible directions from cellular chromatin. Robert Tjian and his colleagues had identified a protein fraction, Sp1, that is required for transcription of SV40 DNA in cell free systems containing RNA polymerase II. This factor was not required for any known cellular promoter. Because of the similarity between the cloned monkey segments and the SV40 control region, we initiated collaborative experiments with Tjian's laboratory to investigate whether transcription from the monkey segments depends, *in vitro*, on Sp1.

Methods Employed:

Methods include radioisotope tracer techniques, preparative ultracentrifugation, DNA-DNA and DNA-RNA hybridization both in solution and with DNA fixed to nitrocellulose filters, column chromatography. Extensive use is made of restriction endonucleases and both preparative and analytical gel electrophoresis for the analysis and preparation of DNA fragments. Specific enzymatic procedures are used for modification or isotopic labeling of DNA fragments. Primary nucleotide sequence determination of DNA fragments is carried out by direct DNA sequencing techniques introduced by Maxam and Gilbert. DNA fragments are purified and prepared in µg quantities by recombinant DNA techniques using *E. coli* K12 cloning systems. The ability of cloned monkey segments to function as sites for initiation of transcription is studied with special recombinant vectors designed for use in animal cells. Desired constructions are made *in vitro* and then amplified in *E. coli* before transfection into mammalian cells. All recombinant DNA experiments are carried out under conditions required by the NIH Guidelines for Recombinant DNA Research as approved by the NIH Biosafety Committee. All our work has been facilitated by extensive use of the NIH computer for storage and analysis of nucleotide sequence data. The mapping of RNA transcripts on DNA templates (including the constructed vectors) is done by the S1 nuclease technique and primer extension. *In vitro* transcription is analyzed in partially

purified cell free systems. Products are analyzed by gel electrophoresis and S1 mapping. Binding of DNA to protein fractions is monitored by DNase I sensitivity and gel electrophoresis.

Major Findings:

A 440 bp fragment of African green monkey genomic DNA shares homology with the transcriptional regulatory region of SV40 and has previously been reported to direct transcription in vivo. The specific monkey segment we are studying in detail (clone 7) was also previously shown to contain a site that is hypersensitive to DNase I within monkey chromatin. The earlier work involved two approaches. The first was to study cellular transcripts that initiate from the genome itself. The second was to study the initiation of transcription from recombinant vectors after transfection into cells in tissue culture. In both instances, we demonstrated transcription going in both directions from within the ori-like region and mapped multiple transcriptional start sites by S1-nuclease and primer extension experiments.

This cellular promoter region is notable for the lack of a TATAAA box which is normally present 25 to 30 bp 5' to the site of transcription initiation by RNA polymerase II. We interpreted these findings to indicate that the SV40-like cellular promoter is a member of a rare class of RNA polymerase II promoters that might play special regulatory roles in cellular metabolism. R. Tjian and his colleague, W. Dynan (University of California, Berkeley), have established an in vitro transcription system from extracts of Hela cells. They obtained several protein fractions, one of which, Sp1, stimulates in vitro transcription from SV40 promoters but not from any cellular promoters previously tested. A collaborative project was established to test the effect of Sp1 on transcription from the SV40-like cellular promoters. Sp1 is required for in vitro transcription from the SV40-like monkey sequence and stimulates bidirectional RNA synthesis. Moreover DNA-footprinting studies show that two regions within the SV40-like genomic fragment bind the factor Sp1 and are protected in the DNAse footprinting experiments. These regions, when fused to the proximal, or TATA-like element of the Herpes thymidine kinase promoter, are able to direct Sp1-dependent transcription in vitro. The finding that Sp1 is capable of productive interaction with sequences taken from a cellular promoter supports the idea that Sp1 may play a role in modulating transcription of a special class of cellular genes.

Significance to Cancer Research:

Our studies deal with the interactions between the viral genome and the genome of a permissive host. They are pertinent to the nature and mechanism of viral-host DNA interaction in permissive and transforming (oncogenic) infections. Further, it is now widely recognized that the genomes of tumor viruses contain sequences homologous to normal host DNA sequences. In the case of RNA tumor viruses these are oncogenes. Our experiments indicate that with some DNA tumor viruses such as SV40, regulatory sequences rather than coding sequences are shared by virus and host. Very recently, the G·C rich repeat that is the transcriptional regulatory signal in SV40 and in the SV40-like monkey sequences has been shown to be associated with several distinct cellular genes. In particular, the expression of a rearranged cellular myc gene seems to depend on such signals. Thus, the special class of promoters we identified may be involved in expression of the myc oncogene in a specific tumor.

Proposed Course of the Research:

No additional work on this system is planned here. The experiments will be continued by Tjian and his collaborators and by Saffer.

Terminate.

Publications:

Dynan, W.S., Saffer, J.D., Lee, W.S., and Tjian, R.: The transcription factor Sp 1 recognizes SV40-like promoter sequences from the monkey genome. Proc. Natl. Acad. Sci. USA (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05244-08 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Organization of Repeated DNA Sequences in African Green Monkeys

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. F. Singer	Chief, Nucleic Acid Enzymology Section	LB	NCI
R. Thayer	Chemist	LB	NCI
A. Maresca	Guest Researcher/Visiting Associate	LB	NCI
S. Contente	Staff Fellow	LB	NCI
G. Humphrey	Guest Researcher	LB	NCI
J. Skowronski	Visiting Fellow	LB	NCI
T. Fanning	Expert	LB	NCI
C. Guenet	Guest Researcher	LB	NCI

COOPERATING UNITS *(if any)*

O. Wesley McBride, Laboratory of Biochemistry, DCBD, NCI

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.8	6.5	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors B

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Two types of highly repeated DNA segments in the genome of the African green monkey (Cercopithecus aethiops) are being studied: (1) Certain satellite DNAs are characterized by long tandem repetitions of species specific sequences and centromeric location. Earlier work indicated that the organization of one such monkey satellite, deca-satellite, is highly polymorphic in individual members of the species and that the amounts of deca-satellite and α-satellite, the major monkey satellite, vary (independently) in individual genomes. In an effort to understand the maintenance of such extensive but variable DNA sequences, analysis of junctions between satellite and unique genomic sequence has been initiated; several such DNA segments have been cloned and characterized. In the junctions, satellite is jointed to low copy number DNA sequences. Homologous low copy number sequences also join a species specific satellite in the human genome. (2) The major primate family of highly repeated long interspersed DNA sequences (called LINE-1, previously KpnI family) includes several thousand 6 kbp long units that terminate in an A-rich stretch. In addition there are more than 10^4 dispersed copies of truncated, rearranged and deleted LINE-1 segements in primate genomes. A 6 kbp sequence compiled from independently cloned and sequenced human and monkey LINE-1 subsegments contains open reading frames totalling about 3.5 kbp. The region containing the open reading frame is conserved in rodents. On the basis of these data we proposed that one or more LINE-1 family members may be functional genes and encode a protein. Therefore the existence of polyadenylated cytoplasmic RNA that might be messenger RNA for the putative genes was investigated. Such a transcript, which represents the sense-strand of the LINE-1 sequence, was detected in a human teratocarcinoma tissue culture cell line. The abundance of the transcript varies markedly with previously described variations in the phenotype of these cells. Antibody prepared against a synthetic peptide whose structure was predicted from the open reading frame, detects an appropriately sized polypeptide in extracts of the teratocarcinoma cells.

GPO 914-918

Project Description

Objectives:

The detailed structure, organization and function of the highly repeated DNA sequences in the primates and other mammals are being studied. These sequences fall into three groups: satellites are long tandemly repeated segments generally concentrated at centromeres, SINES are short repeated segments dispersed throughout the genome, and LINES are long repeated segments also dispersed throughout the genome. We are interested in characterizing the various families of such sequences, establishing their frequencies within the genomes and their functions. Recently it has become evident that many of these sequences are changeable in position within DNA. They also undergo reactions which bring the sequences of the various family members into conformity within the genome of a particular species (concerted evolution). We are interested in studying the mechanisms, the time course and frequency of such changes in order to define the fluidity of primate genomes.

Methods Employed:

DNA is isolated from fresh frozen tissue and from cells grown in tissue culture and purified by standard procedures. DNA structural analysis includes the use of a variety of enzymes, including restriction endonucleases, direct DNA sequencing methods (chemical procedure of Maxam and Gilbert and dideoxynucleotide method), radioisotope tracers, gel electrophoresis, and centrifugation. A variety of nucleic acid hybridization techniques, both in solution and on nitrocellulose are used. DNA fragments and RNAs are purified and amplified by molecular cloning in E. coli K12 host-vector systems. Genomic organization is studied by cloning, blotting and hybridization. Functional aspects focus first on transcription of these sequences both in vivo and in vitro, on translation into protein, and on immunological techniques. For this work RNA is isolated from cells and cloned recombinant cDNA libraries are constructed. Finally, recombinant DNA vectors are constructed and transfected into animal cells to study expression. Somatic cell hybrids constructed by standard techniques are used. Antibodies to synthetic peptides are prepared and characterized by standard techniques. Several different methods have been used to analyze for sequence specific DNA binding proteins in extracts of monkey cells: 1) a competitive assay in which a mixture of radiolabeled fragments including the segment of interest is incubated with extract with or without an excess of the irrelevant fragments (unlabeled) and binding to nitrocellulose is assessed; 2) radiolabeled specific fragment is mixed with cell extract in solution in the presence of increasing amounts of unlabeled E. coli DNA as competitor, and the electrophoretic retardation of the labeled fragment caused by complex formation as a function of E. coli DNA concentration is compared with that obtained upon similar incubation with a non-specific labeled fragment; a specific complex should be more stable than a non-specific one to competition by E. coli DNA. Extensive use is made of computer analysis of DNA sequences. All recombinant DNA experiments are carried out under conditions required by the NIH Guidelines for Recombinant DNA Research as approved by the NIH Biosafety Committee.

Major Findings:

I. Deca-satellite

There are two centromeric satellites described at the molecular level in African green monkeys. Deca-satellite, has a 10 bp repeat unit, represents about 0.5% of the genome and occurs on some but not all of the chromosomes. It is not conserved in humans. α-Satellite has a 172 bp repeat unit, represents as much as 20% of the genome and occurs on all chromosomes (2n = 60). Variously diverged and species specific versions of α-satellite occur in all primates that have been analyzed including humans. Satellite DNAs are among the most species specific sequences known.

The most striking feature of deca-satellite, the centromeric satellite that was discovered a few years ago in this laboratory, is its high polymorphism; deca-satellite probes anneal to sets of repeated restriction endonuclease fragments that differ from individual to individual in the monkey population. Last year's report described experiments aimed at clarifying the structure and organization of deca-satellite sequences and investigating the mechanisms that generate the polymorphism. The results of those experiments suggested that deca-satellite undergoes a continuous reorganization that is the cause of its polymorphism. The contemporary structure of deca-satellite, like that of many other satellites, is consistent with its evolution through alternating cycles of amplification (expansion), deletion (contraction) and mutation. The very drastic polymorphism in sequence and chromosomal distribution that characterizes deca-satellite suggests that the evolutionary processes are now continuing at a rate that is much higher than the rate of fixation. On the basis of these observations, we suggested that the existence of and maintenance of centromeric satellite DNAs rests on the role of the tandem repeats themselves and not on any particular nucleotide sequence arrangement or repeat length. Further, we suggested that these tandem arrays may provide a fertile field for recombination at a critical chromosomal locus. Both the conservation and fluidity of the tandem repeats (but not their specific structures) may be a consequence of the same recombinational events that the satellites foster.

We wished to test the idea that centromeric processes may continually generate or maintain tandem repetitions and that the tandem arrays may also participate in those processes. As an initial experimental approach to this problem, we searched for junctions between satellite and unique DNA sequences. Such junctions are likely to be informative about centromeric structure and processes but have not previously been studied. Moreover, if such junctions are functional then unlike the satellite DNAs they may be conserved in evolution.

As described last year, 200 phage that annealed with deca-satellite were plaque-purified from a random African green monkey genomic library in λ Charon 4A. From these we selected those which annealed only to deca-satellite probe and did not anneal to probes of either α-satellite or total genomic DNA as well as some that annealed weakly to deca-satellite and also annealed to a probe of total genomic DNA: altogether 40 phage were analyzed. We anticipated that at least some of these phage would contain monkey segments composed of

some deca-satellite and adjacent low copy number sequence. Three of these phage appeared to have the desired characteristics and one (λJA) has now been analyzed in considerable detail. The insert (9.4 kbp) contains at most 4 kbp of deca-satellite. Directly abutting it is a region of several kbp which has a low genomic copy number. This is followed, distal to the deca-satellite, by about 2.4 kbp that contains some sequence that is repeated 10^2 to 10^3 times in the monkey genome. At least some of this consists of the so-called evolutionarily conserved sequence, alternating CA (TG) dinucleotides. The analysis of genomic DNA from two monkeys with the subcloned probes from λJA confirmed that the sequence organization bordering deca-satellite in λJA exists in genomic DNA. However, the sequences in the satellite region of λJA do not have the same organization in the genomes as they do in λJA. While this could signal an artefact, it might be expected if a fluid satellite resides at this position. To confirm the association of the λJA sequences with satellite we screened approximately 5×10^5 phage from a different monkey library with the non-satellite region of λJA. Seven phage were isolated. Four of these annealed to a deca-satellite probe and, surprisingly, to an α-satellite probe as well. These findings confirm the association within the monkey genome of the isolated low copy number sequence and satellite DNA. Three of the seven phage did not hybridize with either monkey satellite. We noted that all seven of the phage gave small plaques and proved difficult to grow in liquid culture; very low yields of DNA were obtained. For this reason the structure of only three of the phage, λM8, λM20 and λM31 were analyzed. The purpose of this analysis was to see how clearly these phage matched the genomic organization predicted from the structure of λJA and from the genomic blot experiments. λM8, which contains no satellite sequence has a sequence that is very similar to the non-satellite portion of λJA and contains additional sequences that are distal to the satellite in the genome. Restriction endonuclease mapping and hybridization experiments indicated that the segment cloned in λM8 has the same structure as that in genomic DNA. We can conclude that no significant rearrangements occurred in this portion of the sequence during cloning. Minor differences between λJA and λM8, such as the absence of the CA repeat of λJA at the same position in λM8 could be the result of changes during cloning but could also represent minor differences between multiple copies or multiple alleles of this segment. More significant differences proximal to the region where satellite DNA is joined could be the result of allelic rearrangements of the type expected for satellite either in the monkey cells or in E. coli. λM20 and λM31, both of which contain both satellite and the low copy junction sequences, proved difficult to characterize. DNA-blot analysis of the phage DNA after restriction endonuclease digestion showed that a portion of the low copy junction sequence, deca- and α-satellite all annealed to discrete restriction endonuclease fragments. In some cases all 3 probes annealed to the same restriction fragment indicating that all the sequences are linked. However the digests also indicated that both the monkey segment and the λ-phage cloning vector had undergone extensive rearrangements. This is an interesting observation because analysis of various, often (unrelated) clones from the same monkey library have always been stable.

The subcloned non-deca satellite probes from λJA anneal to human, mouse, and Chinese hamster DNA. Thus the sequences that border deca-satellite in the monkey genome are highly conserved, at least in mammals. We next asked if,

in addition to the sequence conservation, the position of the sequences next to satellite DNA is also conserved. We screened a human genomic library with a non-satellite segment from λJA and isolated 15 positive clones. All of these annealed with monkey probes representing at least about 2 kbp of the monkey junction sequence, indicating that a large portion of the junction segment is conserved intact. Of these, 14 phage annealed to a cloned segment of human satellite III containing a 5 bp repeat unit. Ten of the 14 plaques also anneal to one version of the human α-satellite and one phage did not anneal to any of the human satellite probes tested. As with the monkey clones, these human phage are difficult to grow and yields of DNA are low in spite of the fact that a different λ-phage vector was used to construct the human and monkey libraries. These data suggest that the monkey junction sequence isolated in λJA is associated with satellite in human DNA. These experiments suggest that association with satellite DNA of the low copy number junction sequence we have cloned is conserved in primate genomes. Furthermore, the peculiar characteristics of recombinant phage containing these segments is observed with both human and monkey DNA inserts, suggesting that they may be related to the nature of the primate DNA segments themselves.

II. The LINE-1 Family

The LINE-1 (previously called KpnI) family is a large set of dispersed repeated sequences in primate genomes. An homologous LINE-1 family occurs in rodents (previously called BamHI of MIFI family). The size of the longest family members is between 6 and 7 kbp. The size of the homologous region is about 4 kbp. It lies within the 6 to 7 kbp unit. In contrast, there is little or no homology at the 5'- or 3'-ends of the mouse and primate segments. Earlier structural analysis of cloned family sequences revealed that the LINE families include a complex assortment of members of variable length that share some but not all the sequences present in longer repeated units. Moreover, common sequences within some of these shorter family members are not necessarily colinear but may be either reordered or inverted relative to one another. In primates, it seems that "complete" units are between 6.1 and 6.6 kbp in length and colinear in sequence. No terminal repeats, either direct or inverted, are seen within these full length family members which may or may not be immediately flanked by direct repeats that appear to be target site duplications generated by insertion of a mobile LINE-1 unit into a new genomic locus. At the 3'-terminus (as customarily written), several family members have polyadenylation signals followed by a d(A)-rich stretch; the length and precise sequence of this stretch is variable. At the 5'-end, homology between the full length members starts unambiguously (comparing 6 different family members). Sequences from the 3'-end of the full length LINE-1 are 4 to 5 times more abundant in the genome than those at the 5'-end, approximately 20,000 compared to 4,000 copies, respectively.

The primate L1 sequences that we have determined or have been published represent randomly selected clones. Surprisingly, two cloned human segments (studied by others) have open reading frame (ORFs) over 600 bp long that read 5' to 3' in the designated direction. One of these ORFs is close to the 3'-end of the L1, within the sequences known to be homologous in mouse and primates. Its termination codon coincides precisely with the end of the primate-mouse homology. The sequences of many cloned subsegments of L1 from

human and monkey genomes are available and we positioned these on the restriction endonuclease map. Among these are a few that overlap. We carried out additional sequence analysis in order to bridge the remaining gaps. Consequently, a continuous sequence of a little more than 6 kbp was compiled. In some regions it represents a consensus. We were surprised to find four ORFs totalling almost 3.5 kbp on one strand, starting about 2 kbp from the 5'-end; these ORFs include the shorter ORFs previously noted in cloned subsegments. The apparent discontinuities and reading frame switches that arise from stop codons separating the four ORFs may reflect sequence errors or divergence in the presumably non-coding family members that have been analyzed. Thus the LINE-1 family may include some functional genes that could encode a protein of over 1000 amino acids as well as a large number of pseudogenes. If so, it is remarkably different from other multigene families in two ways. First, the copy number is very high and second, the pattern of truncation is unique. Two major classes of questions emerge from the structural data. One concerns function. Is LINE-1 a family containing genes and pseudogenes? If so, what, where, and when is the significance of the functional genes? The second major class of questions is: What mechanisms explain the amplification, homogenization, dispersal and truncation of LINE family members? If the bulk of the copies are pseudogenes, they are unusual both in their extraordinarily high copy number and in their truncation. In order to ask whether the L1 family contains a functional gene(s) we have begun to search for specific transcripts and related polypeptides. Polyclonal antibodies to three synthetic peptides predicted from the ORFs have been prepared and purified through affinity columns. We chose peptides on the basis of hydrophilicity, presence of a proline residue and confidence in the DNA sequence. The antibodies are being used to screen for antigenic components in the NTera2/D1 cells described below.

We and others demonstrated heterogeneous nuclear transcripts (400 bp to 10 kbp) that anneal to LINE-1 probes in a variety of standard monkey and human cell lines. The size distribution suggests that primary transcripts contain both LINE-1 and unrelated DNA sequences and are the products of RNA polymerase II read-through transcription initiated at unrelated promoters. Both strands of the unit are detected. Our experiments also show that sub-regions along the 6 kbp unit are represented in heterogeneous nuclear RNA in proportion to their copy number in the genome in a number of human and monkey cell lines. Moreover, only a very small percent of the heterogeneous nuclear transcripts are polyadenylated or enter the cytoplasm in most cell lines tested. Uniquely, the human teratocarcinoma line, NTera2D1 (provided by Dr. Peter Andrews, Wistar Institute) revealed an approximately 6.5 kb cytoplasmic polyadenylated RNA. The RNA anneals to probes that span essentially the entire 6 kbp genomic unit. Moreover, it represents only one strand of the LINE-1 unit, the one with the open reading frames and 3' A-rich tail. Thus, it may represent messenger RNA transcribed from one or more genes in the LINE-1 family. The RNA is only a very small proportion of the total $polyA^+$ cytoplasmic RNA. It is most abundant in confluent, piled-up NTera2/D1 cells, just detectable in cells growing at low density and undetectable upon induction with retinoic acid. These experiments suggest that the putative L1 family genes may be specifically expressed in very early embryos.

In order to search for a protein or proteins encoded by LINE-1, antibodies to short synthetic peptides derived from the open reading frames were prepared.

These are being used to screen for reactive proteins in extracts of the NTera2D1 human embryonal teratocarcinoma cell lines. Specifically, antisera were raised in rabbits to three different synthetic peptides: A 15-, 16-, and 18-mer, each chemically coupled to a carrier protein. The peptides were selected based on the likelihood of their immunogenicity as well as a high confidence in the amino acid sequence, i.e., conservation throughout a number of independent primate LINE-1 subclones and in mouse LINE-1 segments. The IgG fraction was purified from both pre-immune and immune antisera on Protein A-Sepharose columns. Immune IgGs were further affinity purified on a corresponding peptide-Sepharose column. Antibody titers and antigen specificity were determined by an enzyme linked immunosorbent assay (ELISA) using uncoupled peptide as bound antigen. Antibodies were successfully raised to each of the three peptide antigens; there ws no cross-reactivity among the three. The antibodies have been employed in indirect immunofluorescence studies on whole, fixed tissue culture cells, immunoblotting of unlabeled whole cell protein extracts, and immunoprecipitation of ^{35}S-methionine labeled cell extracts. Immunoprecipitation has yielded the most promising results. Protein extracted from NTera2D1 cells grown to a confluent, multilayered state and labeled with ^{35}S-methionine for 5 or 24 hours, is reacted with antibody and immune complexes are precipitated with Protein A-Sepharose. After resolution of dissociated complexes on SDS-polyacrylamide gels and autoradiography, a protein band of roughly 150 kD that is specific to one of the three antibodies appears. A protein of this size is consistent with the length of the known open reading frames. Immunoprecipitation of this protein is competed by an excess of corresponding free peptide, but not by unrelated peptide.

L1 probes anneal in situ to many scattered chromosomal locations. In cloned segments they flank genes, occur in introns, and in centromeric satellite DNA. It is not known whether their distribution is random. The idea that the L1 family includes functional genes does not preclude the possibility that some dispersed, non-coding family members (including truncated ones) significantly influence the expression of neighboring genes or chromatin structure. For this reason we initiated a search for protein(s) that bind specifically to L1 sequences. Extracts of monkey and human cells were analyzed for binding activity. Several different techniques were used to try to detect polypeptides that bind to LINE-1 DNA sequences. None of these gave consistently positive results.

Studies on the evolution of the LINE-1 family in various mammals have been continued. Our interest has centered on whether and how species-specific variations in the many LINE-1 family members in a single genome evolve in concert. Studies are now extended to various species in the genus Mus and in the family Felis. A variety of total genomic DNAs from various Mus species have now been surveyed with a LINE-1 probe from the conserved region. Consistent with the occurrence of concerted evolution, prominent differences in restriction endonuclease fragment patterns have been detected. The patterns correlate with evolutionary distance from the laboratory mouse, Mus musculus domesticus.

Future Course of the Work:

I. Satellite - low copy number sequence junctions.

Structural studies in mammalian genomes. The primary nucleotide sequence of the entire segment joining deca-satellite in λJA will be determined. The human recombinant phage will be mapped to determine the arrangement of satellites and the junction sequence. Using subcloned probes and a panel of human/mouse somatic cell hybrids, we can identify how many and which human chromosomes have the junction sequence and its various subportions. We will ask whether cloned mouse segments selected with the junction sequence probes are associated with mouse satellite. If in 3 different species (mouse, human and monkey) the same sequence borders the species specific satellite DNAs, then it is likely to be a pericentric segment. We will also use the genomic extensions represented in λM8 to study the extent of the homologous region in human and mouse. We also plan to study the two monkey recombinant λ-phage that were isolated along with λJA but have not yet had detailed attention.

The nature and significance of the small plaques, low yields and rearrangements observed in the clones containing junction sequences will be analyzed. In particular, attempts will be made to find E. coli host cell strains where the phage may grow more normally. Also, we will try to identify those monkey (or human) DNA segments that are responsible for the poor growth and rearrangements.

Functional studies in mammalian cells. Experiments directed at the following two questions are being planned. (1) Does the junction sequence influence DNA replication? (2) Does the junction sequence foster rearrangements such as tandem reiterations in neighboring DNA? In order to study these questions we will construct appropriate recombinant DNA vectors and transfect them into monkey cells in culture using standard techniques. For example, vectors containing SV40 ori's will be competent for replication upon transfection if a source of T-antigen is supplied. We will measure the relative rates of replication by measuring incorporation of ^{3}H-thymidine into closed circular duplexes and compare the rates with those found with control vectors lacking the junction segments. Should the junction sequences affect the overall replication rate we will try to define the relevant sequence by (1) measuring relative ^{3}H-thymidine incorporation into individual restriction endonuclease fragments of the molecules after brief labeling and (2) by construction of deletions. We will then examine the newly replicated molecules for rearrangements by mapping (1) the population of molecules and (2) recloned representatives.

II. LINE-1 Sequences

Because antibodies to synthetic peptides have frequently proved to recognize the peptide determinants within proteins, we prepared the polyclonal antibodies described above. We will continue to pursue our studies with these antibodies using a wide range of conditions. We may also prepare antibodies to partial peptide products synthesized in E. coli from ORFs cloned in appropriate vectors. If the preliminary results are repeated we will try to use the antibodies to localize the antigen in cells and purify the protein.

Cytoplasmic, polyadenylated RNA from piled-up NTera2/D1 cells is being used for the construction of a cDNA library. Clones annealing to monkey LINE-1 probes will be selected and characterized; clones that include the 5'-end of the 6.5 kb RNA are especially important. If we obtain cDNAs for the 6.5 kb RNA, then its distinctive sequence features may permit isolation and characterization of functional genes. In another approach, the 6.5 kb polyA$^+$ RNA from NTera2/D1 cells will be selected by annealing to probes from the 5'-end of the LINE-1 unit and used in in vitro translation experiments. Hybrid arrest analysis can assist in identifying the putative LINE-1 protein. Also, the antibodies will be tested for their ability to precipitate in vitro translation products.

Because our data suggest that the putative LINE-1 genes may be expressed in early embryos, we will carry out experiments with the mouse LINE-1 family since mice provide suitable experimental material. We now have available many cloned segments of the mouse LINE-1 family. We plan to use these to probe for cytoplasmic polyA$^+$ RNA in mouse teratocarcinoma cells and, by in situ hybridization, for cytoplasmic RNA in early mouse embryos.

Evolutionary studies will be extended to correlate conserved regions of the LINE-1 sequences in various orders and families with functionally important regions as well as to learn something about the timing and mechanism of concerted evolution.

Significance to Cancer Research:

Cancer research has suffered from a lack of basic knowledge about the organization of the eukaryote genome at the molecular level. Methods developed during the last decade have already demonstrated their power to deal with this vast and important unknown. The work we are doing is part of the broad effort to apply new approaches to the elucidation of complex genomes. In particular, we are concentrating on the surprisingly large amount of mammalian DNA included in highly repeated sequences with a capability for mobility and rearrangement in the genome. Work in many laboratories has now shown that genomic rearrangements are often found in tumor cells. It is therefore important to have a more complete picture of recombination in general and, more particularly, of mobile elements in primates.

Another important but still unanswered question about these sequences is their function. Particularly in view of their enormous abundance, the enigma of function is a frustrating puzzle. Speculation ranges from the absence of any function at all to a variety of critical processes. The newly defined hypothesis regarding satellite function can lead to information about the role of centromeres in cell division. The finding that the KpnI family may include functional genes dictates new directions for research, directions that may reveal fundamental information about normal and tumor cells.

We will carry out experiments designed to clone those LINE-1 family members that are functional genes.

Publications:

Maresca, A., Singer, M.F., and Lee, T.N.H.: Continuous reorganization leads to extensive polymorphism in a monkey centromeric satellite. J. Mol. Biol. 179: 629-649, 1984.

Grimaldi, G., Skowronski, J., and Singer, M.F.: Defining the beginning and end of KpnI family segments. EMBO J. 3: 1753-1759, 1984.

Singer, M.F., and Skowronski, J.: Making sense out of LINES - The highly repeated and long interspersed sequences in mammalian genomes. TIBS 10: 119-122, 1985.

Skowronski, J., and Singer, M.F.: Expression of a cytoplasmic LINE-1 transcript is regulated in a human teratocarcinoma cell line. Proc. Natl. Acad. Sci. USA (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05258-06 LB

PERIOD COVERED

October 1, 1984, to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Studies of Eukaryotic Gene Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

B. M. Paterson	Research Chemist	LB	NCI
J. Hammer	Guest Researcher	LB	NCI
J. Eldridge	Biochemist	LB	NCI
A. Seiler-Tuyns	Fogarty Visiting Fellow	LB	NCI
B. Billeter	Visiting Associate	LB	NCI
A. Levi	Visiting Associate	LB	NCI
R. Horlick	Graudate Student	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The cell cycle regulation of structural varients of the mouse histone H4 gene have been analyzed by transfection into L cells with the PSVgpt vector system. Elutriation experiments with the transfected L cells indicate both transcriptional regulation and mRNA stability play a role in the cell cycle dependent expression of the H4 gene. The level of expression and the appropriate regulation of the chicken alpha skeletal and alpha cardiac actin genes in mouse myogenic cells is dependent upon the muscle cell background used for transfection. In contrast the expression of the transfected chicken beta cytoplasmic actin gene decreases during myogenesis in all the muscle cell backgrounds examined to date, in parallel with the endogenous mouse beta actin gene. Promotor exchange studies and nuclear runon experiments suggest the decrease in beta actin expression is controlled at the transcriptional level by a region 3' to the beta actin promotor. The analysis of alpha actin regulation in the different myogenic cell backgrounds is under investigation. To facilitate these regulation studies the complete nucleotide sequence of the chicken alpha caridac actin gene has been determined. There are no significant sequence homologies in the 5' or 3' noncoding regions between the chicken alpha cardiac and alpha skeletal actin genes eventhough both genes are expressed in developing muscle. In constrast, the chicken and human alpha cardiac actin genes show 75% homology in these regions. Cytoplasmic myosin gene of acanthamoeba has been isolated. Sequence analysis has defined the transcriptional unit and demonstrated structurally conserved regions shared with other lower and higher eukaryotic myosin genes. PC 12 cells are induced to undergo neuronal differentiation in response to Nerve Growth Factor. A cDNA sequence and the corresponding gene, induced 50-80 fold in 5 hours after NGF treatment, have been isolated and partially characterized. The gene encodes a polypeptide of 85 kD. Gene encoding the muscle specific isoform of pyruvate kinase has been isolated and all the coding exons defined. Expression studies with the myosin light chain promotors for LC1 and LC3 indicate the promotors do not function out of the context of the entire gene. A gene specific enhancer(s) may control light chain promotor function.

Project Description

Objectives:

I. To prepare ds cDNA probes for the proteins of interest, and to utilize these probes to isolate the genomic sequences for structural studies. These studies primarily involve sequence analysis of all or part of the transcriptional unit for a given gene.

II. To define the transcriptional start sites, splice junctions and termination signals for the genes of interest. This information will be used in a comparative study of differentiation specific and "house keeping" sequences.

III. Some of the ds cDNA probes are to be used in chromosomal localization studies on mouse chromosomes. Several of the structural genes under investigation are highly conserved across species. It is of interest to know if isozymes are proximal or distal on the same chromosome, or on different chromosomes.

IV. When the promotor regions for various genes have been identified, it is intended to analyze promotor function in one of the eukaryotic vector systems now available. We intend to: 1) see if a promotor for a differentiation specific gene, such as α actin, can function when placed in an undifferentiated cell or if differentiation is required for function; 2) define the essential sequence elements for promotor function; 3) determine the role of the intron arrangement in the regulation of gene expression; 4) examine the role of polyadenylation in the regulation of gene expression.

Methods Employed:

Specific ds cDNA probes are prepared either from A^+ mRNA or mRNA fractionated on preparative methylmercury gels. Fractions enriched for a particular mRNA, as judged by analysis in a cell-free protein synthesizing system, are cloned using standard methods. Clones are identified by positive selection and sequence analysis.

The probes are used to screen the charon 4A library of the chicken genome with standard procedures. The distribution of the coding information and its polarity within a given isolate is determined by hybridization with kinased RNA and short cDNA. The 5' proximal and 3' proximal fragments in the appropriate restriction digest are sequenced to clearly determine the end points of the transcriptional unit for each gene. Electron microscopic studies give the preliminary information on the intron-exon pattern and the relatedness of the various isolates for a given gene. Restriction fragments containing regions of interest are subcloned into one of the vector systems for further analysis.

Major Findings:

I. Actin

We have introduced the chicken genes for beta cytoplasmic actin and alpha-cardiac actin into three different murine myogenic cell lines each with a

different developmental histories: the C2 line is derived from adult satellite cells whereas the F3 and B1 cell lines are of embryonic origin. In the C2 line alpha cardiac actin is constituatively expressed at high levels throughout all phases of myogenesis eventhough the endogenous mouse cardiac actin gene is expressed only in differentiated muscle cells. In contrast, chicken alpha actin expression is appropriately regulated in the F3 and B1 lines. This difference in expression is being utilized to define the regulatory regions of the alpha cardiac actin gene and to assay for potential transcriptional activators in the C2 cells. The beta cytoplasmic actin gene is appropriately regulated in all the cell lines examined and expression of the transfected gene decreases during myogenesis in parallel with the endogenous mouse beta actin gene. Promotor exchange and nuclear runon studies suggest the expression of the beta actin gene is transcriptionally regulated by a region 3' to the beta actin promotor. Various minigene constructions are being used to define this region more accurately.

II. Histone H4 Regulation with Cell Cycle

We observe a consistent cell-cycle regulated expression of a mouse histone H4 gene reintroduced into mouse L-cells. The level of expression of the introduced gene is reduced significantly when linkers are inserted in a region corresponding to the 5' non-coding end of the histone H4 gene, but the regulation is conserved. Replacement of the alpha globin promotor (human) with the histone promotor suggests some cell cycle regulation of globin expression in L cells although the level of expression is low. When an intron from the human globin gene is inserted into the H4 coding region, chimeric transcripts are correctly initiated and spliced, but processing at the 3' end is perturbed. The portion of RNA correctly spliced at the 3' end appears cell-cycle regulated, whereas the longer transcripts are not. These longer transcripts are cell-cycle independent in confluent cells. Replacement of the histone specific 3' regulatory element by a fragment containing poly-A addition signals results in very low levels of non-regulated expression of this chimeric gene. We conclude that the cell-cycle regulation of histone expression depends primarily on the integrity of the 3' end of the gene since alterations in the 5' non-coding region or the introduction of an intron does not eliminate cell-cycle regulation, whereas replacement of the element of dyad symmetry with a polyadenylation signal abolishes cell-cycle regulation. Promotor exchange studies suggest transcriptional regulation may play a minor role in regulation.

III. Structure of the Pyruvate Kinase Gene

Characterization of the pyruvate kinase gene is still under way. To date the entire coding region of the gene has been defined with roughly 2300 base pairs of coding region distributed in 10 exons extending over 17-18 kilobases. The large intervening sequence in the 5' non-coding portion of the gene is at least 4 kilobases in length. A 42 base pair oligomer representing the 5' non-coding region of the pyruvate kinase mRNA has been synthesized and subcloned into PBR322 and is being used to screen a size fractionated genomic library for the 5' non-coding exon of the gene.

IV. Acanthamoeba Cytoplasmic Myosin II Gene

We have used a heterologous myosin gene clone to search for the genes of the three Acanthamoeba myosin isoenzymes, myosin IA, IB, and II. The heterologous probe is a 2.7 Kb BamHl fragment of the C. elegans body wall myosin heavy chain gene provided by Jonathan Karn (MRC, Cambridge). This Bam fragment contains coding information for the ATP and actin binding sites as well as the active thiols. We found that this fragment hybridized to discrete DNA fragments in Southern blots of Acanthamoeba DNA and to at least 2 large RNA species (5300 bp and 4250 bp) in Northern blots of Acanthaemoeba poly A + RNA, suggesting that this fragment might well be able to detect the Acanthamoeba myosin heavy chain genes. We screened an Mbol partial-digest genomic library of Acanthamoeba DNA with this probe and selected 40 positive phage. These 40 phage were plague purified and 17 were mapped by restriction enzyme analysis. These 17 phage fall into 4 non-overlapping groups. A representative phage from each group was tested by hybrid selection. The phage from groups 1 and 4 hybrid select a mRNA which translates to give a 185,000 da protein which co-migrates on SDS-PAGE with authentic Acanthamoeba myosin II heavy chain. One phage was characterized in detail and the region corresponding to the active thiol site characteristic of all myosins was sequenced, confirming the identity of the gene. Subsequent sequence analysis has defined the 3' and 5' regions of the gene providing information on the definitive structure of the COOH terminus and the positions of the introns. The precise location of the transcriptional initiation site is under investigation.

V. Myosin Light Chain 1-3 Gene Organization

The myosin light chains 1 and 3 are encoded by a single gene. The organization of the gene has been determined by sequence analysis and contains nine exons spanning 18Kb. Initiation of transcription of LC1 and LC3 starts at different promotors, is developmentally regulated, and processing involves differential splicing. The two adenylation sites on the gene are used randomly in all muscle types examined. Initial attempts with expression studies indicate the individual LC1 and LC3 promotors are not functional out of the context of the entire gene suggesting a gene specific enhancer may be involved in light chain gene expression.

VI. Gene Induction by Nerve Growth Factor

PC12 cells are derived from the adrenal gland of the rat and differentiate into nerve tissue in response to nerve growth factor (NFG). The morphological response to growth factor is not manifest for several days in the presence of NGF; however, new patterns of gene expression are triggered rapidly 24 hours after the application of NGF. We have isolated a cDNA clone and the corresponding gene representing a sequence that is rapidly induced 50-80 fold in response to NGF treatment of the target PC 12 cells. The gene encodes a protein of 85Kd. We are in the process of sequencing the cDNA and gene to define the encoded polypeptide.

Projected Course of Research:

We intend to analyze the organization, structure, and regulation of constitutive and differentiation specific genes.

Publications:

Paterson, B.M., Seiler-Tuyns, A., and Eldridge, J.D.: Expression and regulation of chicken actin genes in avian and murine myogenic cells. In Davidson, E.H. and Firtel, R.A. (Eds.): Molecular Biology of Development. New York, Alan R. Liss, Inc., 1984, pp. 383-394.

Seiler-Tuyns, A., Eldridge, J.D., and Paterson, B.M.: Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells. Proc. Natl. Acad. Sci. U.S.A. 81: 2980-2984, 1984.

Paterson, B.M., and Eldridge, J.D.: Alpha cardiac actin is the major sarcomeric isoform expressed in embryonic avian skeletal muscle. Science 224: 1436-1439, 1984.

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Zehner, Z.E., and Paterson, B.M.: The chicken vimentin gene: Aspects of organization and transcription during myogenesis. Annals of the New York Academy of Sciences (in press).

Paterson, B.M., Eldridge, J., and Seiler-Tuyns, A.: Regulation of actin gene expression: Analysis of potential regulatory regions. J. Cell. Biochem. (in press).

Eldridge, J., Zehner, Z., and Paterson, B.M.: The complete nucleotide sequence of the chicken cardiac alpha actin gene: No strong sequence homologies in the promoter and 3'-translated regions with the chicken skeletal alpha actin gene. Gene (in press).

Levi, A., Eldridge, J., and Paterson, B.M.: Molecular cloning of a gene sequence regulated by nerve growth factor. Science (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05262-05 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Eukaryotic Gene Regulation and Function: The Metallothionein System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Dean H. Hamer	Research Chemist	LB	NCI				
C. Schmidt	Chemist	LB	NCI	C. Seguin	Guest Researcher	LB	NCI
B. Felber	Visiting Fellow	LB	NCI	D. Thiele	Guest Researcher	LB	NCI
A. Leone	Visiting Associate	LB	NCI	C. Wright	Staff Fellow	LB	NCI
M.J. Walling	Microbiologist	LB	NCI				

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.0	7.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The regulation and function of metallothioneins are being analyzed by molecular genetic and biochemical approaches in both mammalian cells and yeast. Human metallothionein genes exhibit cell-specific expression that can be altered by the ras oncogene. The specificity of expression is correlated with differential metal-binding properties of the proteins. Attempts are in progress to detect and isolate the cellular factors involved in regulation by heavy metals and other agents. A copper-inducible metallothionein-like protein of yeast has been demonstrated to serve two functions: copper detoxification and negative autoregulation of its own expression. Both functions are complemented by mammalian metallothionein demonstrating the functional analogy between the higher and lower eukaryotic systems.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

We wish to understand how eukaryotic genes are activated or repressed in response to changes in the cellular environment and how the resulting gene products allow the cell to adapt to its altered milieu.

Methods Employed:

Our general strategy is to use recombinant DNA, biochemical and genetic techniques to identify the regulatory DNA sequences and the cellular factors with which they interact and to characterize the physiological roles of the gene products.

Major Findings:

I. Regulation and Function of Mammalian Metallothionein

Metallothionein (MT) synthesis is an especially useful system for studying eukaryotic gene regulation and function. These small, cysteine-rich proteins have been found in all eukaryotes examined, ranging from yeast to man, and are expressed in many different organs and cell types. MT gene expression is inducible by the same heavy metals to which the MT proteins bind. This homeostatic regulatory mechanism plays a critical role in detoxifying toxic ions, such as cadmium and mercury, and may also play a role in the metabolism of essential metals such as copper and zinc. In addition, metallothioneins exhibit distinct patterns of expression during development and differentiation.

A. Search for Regulatory Factors

We previously demonstrated, by mutagenesis and gene transfer experiments, that a duplicated upstream sequence is responsible for the heavy metal induction of mouse MT-I gene transcription. We also showed, by an *in vivo* competition assay, that these sequences interact with positively-acting cellular factors. We are now attempting to detect these factors *in vitro* and to purify them. Initial attempts using affinity chromatography, *in vitro* transcription, nitrocellulose filter binding and the band shift method were all unsuccessful. More recently we have had preliminary success with the elegant exonuclease III footprint assay devised by Dr. Carl Wu in the Laboratory of Biochemistry. Attempts to fractionate the binding activity are in progress.

B. Structure and Developmental Regulation of Human MT Genes

Human MTs are encoded by a complex multigene family that includes at least five functional members clustered on chromosome 16. Two MT-I isoform genes, hMT-Ie and hMT-If, were cloned and sequenced in their entirety. These genes are highly homologous to one another and encode proteins that differ by only four amino acids. Both genes are functional

as shown by their transcription in cultured cells and by their ability to render transfected cells resistant to cadmium toxicity.

The expression of these closely related isoform genes has been studied in a variety of cultured cell types and in liver biopsy samples. We find that the genes are expressed in a highly specific fashion in that only a single MT-I isoform is transcribed in most differentiated cell types. Only in nondifferentiated cells (teratocarcinomas and certain other tumors) is coexpression observed.

C. MT and Metal Metabolism

The highly specific pattern of human MT-I gene expression led us to speculate that different MT isoforms might play subtly different roles in metal metabolism. To test this, we inserted the hMT-II, hMT-Ie and hMT-If genes into a bovine papilloma virus vector and introduced the recombinants into cultured mouse cells. The resulting transformants greatly overproduce the MT encoded by the cloned gene; thus, we can compare metal metabolism in isogenic cell lines that differ only in the MT isoform that is produced. We find that each MT binds different heavy metals in distinctly different stoichiometrics. For example, hMT-Ie-producing cells cultured in media with equimolar copper and zinc accumulate eight fold more zinc than copper, whereas hMT-If producers accumulate equal amounts of the two ions. This differential metal binding may reflect the necessity for different tissues and organs to handle different transition elements. We are studying the structural basis of this phenomena by constructing hybrid proteins carrying different domains from the isoform genes.

D. Oncogenes and MT Regulation

During the course of our developmental studies we made an unexpected observation: cell lines expressing activated ras oncogenes have high constituitive levels of MT gene transcription. The effect appears to be specific since cells expressing other oncogenes, such as SV40 T-antigen, show normal patterns of MT regulation. We are investigating the mechanism of this phenomenon by treating normal cells with cAMP, phorbol esters, growth factors and other agents that are thought to be effected by ras expression. We also intend to determine which MT control sequences respond to ras by transfection experiments. It is tempting to speculate that the ras proto-oncogene plays a role in MT gene regulation during normal development and differentiation.

II. Yeast Copperthionein Model System

The yeast Saccharomyces cerevisiae synthesizes a small, cysteine-rich protein that binds copper ions. The synthesis of this protein, which is encoded by the CUP1 locus, is dramatically increased when copper is added to the culture medium. The tremendous genetic and technical advantages of yeast as an experimental organism make this an attractive model system to study the function and regulation of a metallothionein-like protein.

A. Function and Autoregulation of Copperthionein

We constructed haploid and diploid yeast strains in which the endogenous CUP1 gene is completely deleted and replaced by a yeast selectable marker. Such strains are highly sensitive to copper poisoning, therefore confirming the role of copperthionein in heavy metal detoxification. However, under standard laboratory conditions they grow normally, can participate in all phases of the yeast life cycle, and accumulate normal levels of total cell copper and of a copper enzyme. This shows that copperthionein does not play any essential physiological role, at least under laboratory conditions.

To investigate the possible role of copperthionein in regulating its own expression, we constructed a CUP1-galK fusion gene that contains CUP1 regulatory and promoter sequences but no coding sequences. The expression of this gene was studied in strains lacking or retaining the endogeneous CUP1 structural gene. Suprisingly, strains that fail to synthesize copperthionein protein transcribe the fusion gene at a high constuitive level even in media with no exogeneously added copper. The ability of copperthionein to negatively autoregulate its own expression may play an important role in copper homeostasis in yeast.

B. Transcriptional Regulation

The CUP1-galK fusion gene has been used to study the cis-acting control sequences responsible for regulation by copper. An extensive set of 5' deletions, 3' deletions and linker scanner mutants have been constructed and analyzed. In addition, synthetic oligonucleotides corresponding to the control region have been synthesized and placed upstream of a heterologous gene. This work has revealed a complex set of both positive and negative regulatory signals. The positively-acting sequences are repeated and must be present in more than one copy to be effective.

C. Yeast Model for Menkes' Disease?

We have also isolated trans-acting mutants with altered patterns of CUP1 gene expression. One such mutant shows high levels of basal CUP1 transcription, is sensitive to copper poisoning, and takes up increased amounts of ^{64}Cu from the medium. These phenotypes are remarkably similar to those exhibited by cells from patients with Menkes' disease, an inherited abnormality of copper metabolism in man. Classical genetic analysis shows that all three phenotypes are controlled by a single, semidominant gene. A gene that supresses the copper-sensitive phenotype has been cloned and is being sequenced.

D. Structure-Function Relationships in Copperthionein

We are interested in the structural basis of the detoxifying and autoregulatory functions of copperthionein. As a first step, we collaborated with Dr. D. Winge to purify copperthionein to homogeneity. The isolated protein contains 8 atoms of copper per molecule. The apoprotein is also

capable of tightly binding to silver (8 atoms per molecule), cadmium and zinc (both 4 atoms, per molecule). Thus the yeast protein, like the mammalian MTs, binds to different heavy metals in distinct configurations. The complete amino acid sequence of the protein was determined. Unexpectedly, the first eight amino acids predicted from the DNA sequence are lacking from the purified protein. The processed sequence is unusual in its high proportion of aromatic and hydrophobic residues. To determine the physiological importance of this processing step, we have used oligonucleotide-directed mutagenesis to obtain point mutations in the leader sequence. We are also constructing mutants in the conserved lysine-rich linker sequence and in the metal-binding sites.

E. Mammalian Metallothionein is Functional in Yeast

Two monkey MT cDNAs have been placed under control of the CUP1 promoter and introduced into yeast strains carrying a deletion of the endogenous copperthionein gene. The monkey metallothioneins complement both known functions of the yeast gene, namely copper detoxification and autoregulation. The MT-like proteins of higher and lower eukaryotes are therefore functionally analogous despite their dissimilar sequences.

Publications:

Schmidt, C.J., Hamer, D.H., and McBride, O.W.: Chromosomal location of human metallothionein genes: Implications for Menkes' Disease. Science 224: 1104-1106, 1984.

Hsiung, N., Fitts, R., Strath, W., Milne, A., and Hamer, D.H.: Efficient production of Hepatitis B surface antigen using bovine papilloma virus-metallothionein vector. J. Mol. Appl. Gen. 2: 497-506, 1984.

Seiler-Tuyns, A., Pantazis, P., Bonner, W., Hamer, D., and Kumar, A.: A mouse histone H4 gene carried by an SV40 vector is accurately expressed in infected monkey cells. DNA 3: 215-222, 1984.

Carter, A.D., Felber, B.K., Walling, M.-J., Jubier, M.-F., Schmidt, C.J., and Hamer, D.H.: Duplicated heavy metal control sequences of the mouse metallothionein-I gene. Proc. Natl. Acad. Sci. USA 81: 7392-7396, 1984.

Cereghini, S., Saragosti, S., Yaniv, M., and Hamer, D.H.: SV40-α-globulin hybrid minichromsomes: Differences in DNase I hypersensitivity of promoter and enhancer sequences. Eur. J. Biochem. 144: 545-553, 1984.

Seguin, C., Felber, B.K., Carter, A.D., and Hamer, D.H.: Competition for cellular factors that activate metallothionein gene transcription. Nature 312: 781-785, 1984.

Leone, A., Pavlakis, G.N., and Hamer, D.H.: Menkes' disease: Abnormal metallothionein gene regulation in response to copper. Cell 40: 301-309, 1985.

Lassar, A.B., Hamer, D.H., and Roeder, R.G.: Stable transcription complex on a class III gene in a minichromosome. Molec. Cell. Biol. 5: 40-45, 1985.

Hamer, D.H., Thiele, D., and Lamont, J.: Function and autoregulation of yeast copperthionein. Science 228: 685-690, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05263-04 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Eukaryotic Chromatin Structure and Gene Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Carl Wu	Visiting Associate	LB	NCI
Thomas Paisley	Biologist	LB	NCI
Allan Hansell	IPA	LB	NCI
Barbara Walker	Staff Fellow	LB	NCI
Barbara Wood	Laboratory Worker	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	4.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The sequential arrangement of nucleosomes along the chromatin fiber is punctuated by highly nuclease-sensitive sites. We previously mapped such sites to the 5' terminus of several heat shock genes in Drosophila by a novel indirect end-labeling technique. Such preferentially accessible sites in chromatin may function as points of entry to the DNA for RNA polymerase and control proteins. We have developed an exonuclease protection technique for mapping protein binding sites in chromatin, and have found two such sites for both the hsp 82 and hsp 70 genes. Site I is present before and after heat shock gene activation, and covers the TATA box sequence, whilst site II surrounds the upstream heat shock control element and appears only during heat shock. We suggest that heat shock genes are activated by the sequential binding of at least two protein factors, and we have developed new chromatin-binding and DNA-binding procedures to detect these proteins in crude nuclear extracts. We are now using these procedures as assays for the purification of the two proteins using standard column chromatography and high performance liquid chromatography.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

A knowledge of gene regulation is fundamental to an understanding of eukaryotic development and differentiation. We study gene regulation by probing the structure of DNA associated with proteins in chromatin. We have shown that the 5'-terminal and flanking sequences of Drosophila heat shock genes are uniquely accessible in chromatin to a nucleolytic probe, DNase I. We aim to analyze further the structure and function of nuclease hypersensitive sites in chromation.

Major Techniques Employed and Major Findings:

We have recently developed an exonuclease protection technique for mapping sequences in chromatin onto which regulatory proteins are bound. The technique involves simultaneous digestion of native chromatin with a restriction endonuclease and an exonuclease, and analysis of the cleaved gene fragments on Southern blots. The method reveals two protein-binding sites amidst the hypersensitive region at Drosophila heat shock gene promoters. For the hsp82 gene, one exonuclease-resistant site (-17 to -39) surrounding the TATA box sequence is observed independent of heat shock induction, whereas a second site (-50 to -86) is found to be heat shock dependent. This site covers the minimal sequence element shown by Pelham to be required for heat shock inducible transcription. Essentially similar results were obtained for the hsp70 gene.

We propose that two different nonhistone proteins bind to the observed sites in chromatin. Binding of one specific protein to the TATA box could be responsible for the creation of the DNase I hypersensitive site at the 5' end of heat shock genes, setting the stage for the binding of a second protein (heat shock activator protein, HAP) to the sequence at -50 to -86. RNA polymerase II is directed to transcribe heat shock genes only after both proteins are bound.

We are trying to purify HAP. We can show that HAP is present in crude extracts of heat shocked cells by means of a highly sensitive, in vitro assay based on chromatin binding and exonuclease protection. We use nuclei isolated from non-shocked Drosophila embryos as a substrate for protein-binding, and incubate the nuclei with a 0.4M NaCl nuclear extract from heat shocked embryos or cultured cells. HAP in the extract is able to bind to its proper target in native chromatin, as evidenced in the resistance to exonuclease digestion in the -50 to -86 region of the hsp82 gene. Its binding activity can be destroyed by trypsin or proteinase K treatment, but not by digestion with RNase or DNase. By means of a DNA competition assay, we can show that HAP is able to interact directly with purified DNA containing its target sequence, since pre-incubation of active extracts with that DNA can inhibit subsequent binding to chromatin. Similarly, we also show that HAP can bind to the upstream sequences of other members of the heat shock gene family.

The binding strengths of HAP for the upstream sequences of the heat shock gene family are unequal, and are strongest for the hsp82 gene. Previous work from many laboratories has shown that the hsp82 gene is most easily activated. Hence, the data suggest a hypothesis for the activation of the gene family based on

differential HAP binding affinities. Further work is needed to elaborate the connection between activation kinetics and binding affinity. These and other experiments that are important for understanding the mechanism of activation of heat shock genes will become feasible when HAP factor is purified to homogeneity.

Significance to Cancer Research:

Our work contributes to a broad effort in the study of gene regulation during normal development and differentiation, and will serve as a basis for the study of aberrant cellular functions which result in neoplasmia.

Proposed Course of Research:

We plan on extending our exonuclease protection studies to mammalian genes in chromatin. We plan to isolate the proteins responsible for the binding to the TATA box and the upstream control element in the Drosophila heat shock genes by incubating crude extracts from activated cells with chromatin or free DNA from normal cells, and assaying for specific binding by exonuclease protection. We also plan to study the functional properties of the protein factor, when partially purified, by using our nuclear transcription assay, and by in vitro transcription using cell extracts.

Publications:

Wu, C: Activating protein factor binds in vitro to upstream control sequences in heat shock gene chromatin. Nature 311, 81-84 (1984).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05264-04 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of a Mouse Repetitive Gene Family

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Kira K. Lueders	Chemist	LB	NCI
J. Mietz	Microbiologist	LB	NCI
E.L. Kuff	Chief, Biosynthesis Section	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

We have previously characterized a family of genetically distinctive endogenous retrovirus-like elements which are present in multiple copies in the mouse and other rodent genomes. The bulk of the intracisternal A-particle (IAP) gene family in Mus musculus consists of 7.2 Kb long elements. Deleted forms of these elements have also been described, and the majority of IAP sequences involved in transpositions have been deleted forms. We are now studying a particular subset of deleted IAP genes designated type II IAP genes which are characterized by an insertion (IIins). Clones containing IIins sequences were isolated from a mouse genomic DNA library. Eleven such clones also reacted with type I IAP sequences, suggesting most IIins sequences in the genome occur as parts of type II IAP genes. The IIins sequences are interspersed and are absent or not amplified outside Mus musculus and closely related species. The IIins is 300 bp long and shows 10% divergence among three members sequenced. Type II IAP genes have at their 5' end a region of 74 bp which is duplicated and contains a core enhancer sequence. IIins begin and end at precisely the same point in two subclasses of type II IAP genes, making it likely they derived from a common progenitor. Formation of type II IAP genes probably involved multiple recombinational events. The majority of type II IAP genes appear to be associated with other repeats. Specifically, 11 of 12 type II IAP clones also contained truncated L1 family members. Only 3 of 29 type I IAP clones contained these repeats. Major transcripts of IIins were associated with IAP sequences. Only the type IIA and IIB IAP genes were transcribed. The lack of type IIC gene transcription may result from their association with the L1 repeats which are highly methylated and poorly transcribed. An inhibitory effect of flanking DNA on provirus transcription has been noted by others.

Project Description

Objective:

To study the consequences of the presence of repetitive sequences in the genome, particularly the relationship between the reiterated endogenous retrovirus-like elements of the IAP gene family and normal development and/or neoplastic transformation. Specifically, to determine whether type II IAP genes have a functional role in mouse cells and are expressed as protein products.

Methods Employed:

Culture of animal and bacterial cells; recombination and cloning of specific eukaryotic and viral sequences in plasmids, lambda phage; analysis of DNA components by restriction endonuclease cleavage, electrophoresis, and blot hybridization; isolation of DNA by CsCl banding; electron microscopy of DNA heteroduplexes; nucleotide sequencing by the Sanger dideoxy method; transfection of animal cells with cloned DNA using the CaP precipitation procedure; S1 mapping of transcripts.

Major Findings:

IAP genes represent a family of genetically distinctive endogenous retrovirus-like elements which are present in 2000 copies in the genome of Mus musculus. In the past our studies have dealt with the bulk of the IAP gene family which consists of 7.2 Kb long elements; these are colinear with the genomic RNA of the particles which codes for the IAP main structural protein p73. These elements have been designated type I IAP genes. Deleted forms of these elements have also been described. The majority of IAP sequence element transpositions have involved deleted forms.

We are now studying a particular subset of deleted IAP genes which have been shown to be amplified in myeloma cells to determine whether these elements have a functional role and what is the effect on cells of amplification and transposition of these sequences and of their potential protein products. These IAP elements have been designated type II IAP genes. They contain a characteristic insertion (IIins) and have been divided into three classes A, B, and C on the basis of the sizes of the deletions which they contain.

I. Characterization of Type II IAP Genes

We have isolated clones containing sequences homologous to IIins from a mouse embryo DNA library. The number of plaques which reacted with this probe (0.6%) indicates the copy number is probably higher then originally estimated by others. Eleven such clones also reacted with type I IAP sequences, suggesting most IIins sequences in the genome occur as parts of type II IAP genes.

Southern blot hybridization of IIins to mouse genomic DNA digests shows patterns typical of a family of interspersed sequences. There is no evidence for tandem repeats of these sequences. Hybridization of IIins probe to neterologous genomic DNAs indicates this sequence is not detectable

outside Mus musculus and closely related mouse species, sugggesting that there are no related sequences present, or more likely, that there is no amplification.

II. Sequence Analysis

Three isolates of IIins as well as associated IAP sequences have been sequenced. The IIins sequence is 300 bp long and shows about 10% divergence among the three members. Sequencing of regions of the IAP genes upstream and downstream of IIins has revealed several interesting features: (1) a 74 bp region at the 5' end of type II IAP genes is duplicated. This region contains a core enhancer sequence. A type IIA IAP gene contains two copies of this 74 bp region while a type IIC gene contains four copies. We found that another deleted IAP gene isolated and sequenced in another laboratory also has this region duplicated; (2) In addition to a major deletion of 650 bp 5' to IIins, several 50 bp deletions are also present; (3) The IIins begin and end at precisely the same point in the flanking type I IAP gene sequence, making it likely that IIA and IIC forms are derived from a common progenitor sequence; and (4) A 66 bp IAP sequence immediately 3' to IIins is derived from a region further downstream than that which follows, suggesting that formation of the type II IAP genes involved multiple recombination events. Integration into a retroviral genome may have been responsible for the amplification of IIins in Mus musculus.

III. Association Between Type II IAP Genes and Other Repeats

In some genomic digests probed with IIins, strongly reacting fragments with sizes larger than type II IAP genes were seen, indicating that there are multiple units with similar flanking sequences. Heteroduplex analysis of several type IIC clones in phage confirmed that common flanking sequences were present. Hybridization of the phage clones with labeled genomic DNA indicated all of them contained highly repetitive (>20,000 copies) sequences. Further analysis using probes representing previously identified repetitive families showed that 11 or 12 type II IAP clones also contained truncated sequences of the mouse L1 family. Many clones also reacted with the mouse B1 and B2 family probes. Three DNA fragments carrying L1 repeats were isolated from one phage clone and further characterized by electron microscopy after subcloning in plasmid. These represented multiple copies of overlapping elements; their restriction maps deviated considerably from the concensus map for the L1 family.

IV. Expression of Type II IAP Genes

Major transcripts of IIins sequences were associated with IAP sequences. Transcripts of both type IIA and IIB IAP genes were detected and were more abundant in transformed cells (myeloma) than in normal cells (thymus).

Significance for Cancer Research

Type II IAP genes are expressed and transposed in transformed cells. They are a potential source of genetic variation in the mouse and could play a role in tumor development and/or progression.

Proposed Course of Research:

Type II IAP sequences will be tested for their ability to function as protein coding sequences. Although these IAP genes contain deletions which eliminate most of the sequences which code for the stuctural protein of the particle, it is conceivable that they contain alternate coding sequences with an open reading frame. A precedent for production of novel protein products from deleted IAP genes exists (see Kuff).

Vectors containing the bacterial gene chloramphenicol acetyl transferase (CAT) have been used in a transient expression assay to measure promoter and enhancer activity after transfection into a variety of mammalian cells. We will test the 74 bp region which is duplicated in the type II IAP genes for ability to enhance gene transcription in this system.

Publications:

Lueders, K.K., Fewell, J.W., Kuff, E.L., and Koch, T.: The long terminal repeat of an endogenous intracisternal A-particle gene functions as a promoter when introduced into eucaryotic cells by transfection. Mol. Cell Biochem. 4: 2128-2135, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05265-03 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Cytoskeletal Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. Wagner	Guest Researcher	LB	NCI
J. George	Technician	LB	NCI
N.-D. Vu	Staff Fellow	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Macromolecular Interactions Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Interactions between cytoplasmic myosin and actin filaments are thought to be responsible for a variety of motile activities in nonmuscle cells. As in muscle contraction, hydrolysis of ATP by myosin provides the required energy. While the actin-activated ATPases of vertebrate smooth muscle and nonmuscle myosins are regulated by phosphorylation of their 20,000-dalton light chains, we have found that their mechanisms of activation are very different. An electrophoretic method was developed to separate thymus myosin with two phosphorylated light chains from unphosphorylated myosin and myosin with one phosphorylated light chain. This technique was used to show that the two heads of thymus myosin are phosphorylated randomly. This apparent lack of cooperativity contrasts with the reported ordered phosphorylation of gizzard smooth muscle myosin. The actin-activated ATPase of thymus myosin was found to increase linearly with the fraction of light chain phosphorylated. Since the two heads of thymus myosin are phosphorylated randomly, this linear correlation shows that phosphorylation of one head of thymus myosin stimulates the actin-activated ATPase of that head independent of the phosphorylation of the second head. In contrast, both heads of gizzard myosin must be phosphorylated for actin to activate the ATPase of either head. Analysis of the dependence of the ATPase activity of unphosphorylated thymus myosin on actin concentration showed that phosphorylation does not alter the maximum rate of ATP hydrolysis, but rather it causes a 15-fold increase in the apparent affinity of this myosin for actin. In the presence of tropomyosin, phosphorylation causes only about a 2-fold increase in affinity of thymus myosin for actin. Phosphorylation of gizzard myosin, on the other hand, causes a large increase in the maximum rate of ATP hydrolysis and only a small change in affinity of this myosin for actin.

Project Description

Objectives:

Our general goals are to understand the roles of cellular and smooth muscle actins and myosins, their interactions with each other and with the cell membrane, the regulation of these interactions, and how they relate to the various motile activities of cells and to the regulation of smooth muscle contraction.

Methods Employed:

Standard protein isolation techniques are used to prepare actin, myosin, myosin light chain kinase, and other cytoskeletal proteins from various types of muscles and from thymus and brain. Characterizations of their interactions are done using enzymatic assays, fluorescence spectroscopy, ultracentrifugation, and electron microscopy. T-lymphocytes made permeable by incubation with detergent are used to examine the role of these cytoskeletal proteins in the capping of cell surface receptors.

Major Findings:

During the past year we have concentrated on determining the mechanism of regulation of the actin-activated ATPases of vertebrate smooth muscle and nonmuscle myosins. In addition to their well-established roles in muscle contraction, actin and myosin appear to be responsible for a large variety of cellular motile activities, e.g. cell division and capping of cell surface proteins. The hydrolysis of ATP by myosin provides the required energy. An in vitro analog of this interaction is the actin-activated ATPase of myosin. While the actin-activated ATPases of vertebrate smooth muscle and nonmuscle myosins are regulated by phosphorylation of their 20,000-dalton light chains, we have found that there are significant differences in their mechanisms of activation.

We have developed a method to separate myosins which differ in the number of light chains phosphorylated. When electrophoresed under nondissociating conditions myosins with two phosphorylated light chains migrate more quickly than myosins with one phosphorylated light chain which migrate only slightly faster than the unphosphorylated myosins. This electrophoretic technique was used to determine the order of phosphorylation of a nonmuscle myosin isolated from calf thymus. Both monomeric and filamentous thymus myosin appear to be phosphorylated randomly. This contrasts with the reported ordered phosphorylation of filamentous gizzard myosin. A result which we have confirmed using nondissociating gel electrophoresis. As we reported previously, there is a linear relationship between the extent of phosphorylation of the thymus light chains and stimulation of the actin-activated ATPase. Since the two heads of thymus myosin are phosphorylated randomly, this linear relationship shows that phosphorylation of one head of thymus myosin stimulates the actin-activated ATPase of that head independent of the phosphorylation of the second head. In contrast, both heads of gizzard myosin must be phosphorylated for the ATPase of either head to be activated by actin.

We have found that light chain phosphorylation regulates the actin-activated ATPase of thymus myosin not by increasing the maximum rate of ATP hydrolysis

but rather by causing about a 15-fold increase in the apparent affinity of this myosin for actin. When actin complexed with skeletal muscle tropomyosin was used, the maximum ATPase rates remained about the same, but there was only about a 2-fold difference in affinity. In contrast, phosphorylation of gizzard myosin affects primarily the maximum rate of ATP hydrolysis and not the apparent affinity of this myosin for actin. Thus light chain phosphorylation appears to regulate actin-activated ATPases of thymus and gizzard myosin by very different mechanisms.

The high actin-activated ATPase activities of unphosphorylated thymus myosin indicate that there must be some way of inhibiting the interaction of this myosin with actin. We have used regulated actin (actin plus skeletal muscle troponin-tropomyosin) as a model to examine the effect of thin filament regulatory proteins on the actin-activated ATPase of thymus myosin. At moderate ionic strengths, the regulated actin-activated ATPase of phosphorylated thymus myosin was Ca^{2+} insensitive. However, in the absence of Ca^{2+}, there was no significant stimulation of the ATPase of the unphosphorylated myosin. Thus in the presence of thin filament regulatory proteins, phosphorylation can cause a large increase in the ATPase activity of thymus myosin.

Significance to Biomedical Research and the Program of the Institute:

Cell movement, endocytosis, capping of cell surface receptors, cell division and a variety of other cellular activities are thought to rely on forces generated by the interactions of cytoplasmic actins and myosins. These interactions are in part regulated by Ca^{2+}. The actin-activated ATPase of gizzard myosin has been used as a general model for the interaction of smooth muscle and nonmuscle myosins with actin. The experiments reported here indicate that the interactions of mammalian cytoplasmic myosins with actin are regulated very differently than those of gizzard myosin.

Proposed Course:

We intend to continue characterizing the effects of light chain phosphorylation on the interaction of thymus myosin with actin. The differences in the actin-activated ATPases of unphosphorylated and phosphorylated thymus myosins appear to be too small for this phosphorylation to be an effective regulatory system. This suggests that may be another regulatory system in these cells. While nonmuscle cells contain tropomyosin, there is currently no evidence that they contain troponin-like proteins. We are going to try to isolate thin filament regulatory proteins from the thymus.

Soluble fragments of muscle myosins have been invaluable in the determination of the kinetics of the actomyosin ATPase and in the study of the interaction of these myosins with the thin filament. We will try to make heavy meromyosin and myosin subfragment-1 from thymus myosin. Neither of these fragments has been prepared from a nonmuscle myosin. If they can be prepared, they will allow us to determine which steps in the kinetic cycle are regulated by light chain phosphorylation. These subfragments will also be very useful in the characterization of thin filament regulatory proteins and will provide information on the interaction between the two heads and on the interaction of the heads with the myosin rod.

We are going to examine the effect of light chain phosphorylation on actin-activated ATPase of calf aorta myosin to determine if the interaction of this mammalian smooth muscle myosin with actin is regulated like that of calf thymus or like that of turkey gizzard. There is evidence from intact mammalian smooth muscles that in addition to light chain phosphorylation there is some other type of Ca^{2+} dependent regulatory system.

Capping of T-lymphoma cell surface proteins by concanavalin A will be used as a model system to try to establish the function of various cytoskeletal proteins. Both intact cells and cells made permeable by detergent will be used. While cell surface proteins of these detergent treated cells still cap in response to concanavalin A binding, it appears possible to diffuse large macromolecules, i.e. antibodies and myosin light chain kinase, into them. This system should allow us to directly examine the roles of myosin in this particular motile process.

Publications:

Mendelson, R.A., and Wagner P.D.: X-ray scattering by single-head heavy meromyosin: Cleavage of the myosin head from the rod does not change its shape. J. Mol. Biol. 177: 153-171, 1984.

Wagner, P.D.: Effect of skeletal muscle myosin light chain 2 on the Ca^{2+}-sensitive interaction of myosin and heavy meromyosin with regulated actin. Biochemistry 23: 5950-5956, 1984.

Wagner, P.D., Vu, N., and George, J.N.: Random phosphorylation of the two heads of thymus myosin and the independent stimulation of their actin-activated ATPases. J. Biol. Chem. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05266-03 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of the Immunoglobulin Gene Family

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Cary Queen	Senior Staff Fellow	LB	NCI
J. Foster	Guest Researcher	LB	NCI
J. Stafford	Microbiology Technician	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Macromolecular Interactions Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are studying the regulation of expression of the immunoglobulin gene family by attempting to answer two questions: (1) why do only cells of the B-lymphoid lineage synthesize immunoglobulins, (2) how do these cells transcribe only one or a few immunoglobulin genes, while leaving hundreds of other, similar immunoglobulin genes inactive? Our approach to these questions is to insert a cloned, rearranged kappa light chain gene into a plasmid in various configurations, to transfect the plasmid into various types of cells, and to determine whether the transfected gene is transcribed. We have shown that the complete kappa gene is transcribed after transfection into antibody-producing myeloma cells but not in non-lymphoid 3T3 or L cells. Hence the different cell types are able to appropriately regulate the kappa gene even when not in its usual chromosomal environment. By deleting different parts of the cloned gene, we have shown that certain sequence elements actually downstream of the promoter are necessary for its transcription in myeloma cells. We have localized the down-stream element to a 200 base pair region of DNA and have shown that it is an enhancer. We have recently shown that both the enhancer and the promoter are cell-type specific, that is, they function in lymphoid cells but not in non-lymphoid cells.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To study the regulation of the immunoglobulin gene family. In particular, to learn about the DNA sequences and protein factors that activate expression of immunoglobulin genes.

Methods Employed:

The project involves in vitro modifications of a cloned immunoglobulin gene. The modified genes are transfected into cultured cells of various types by the DEAE-dextran method. RNA is extracted from the cells and assayed by the S1 nuclease method to determine whether the immunoglobulin gene is transcribed.

Major Findings:

Regulation of the Immunoglobulin Gene Family

Each mammalian cell contains several hundred immunoglobulin genes, which encode the proteins that constitute antibodies. Only cells of the B-lymphocyte class (B-lymphoid cells) express these genes, that is, transcribe them into RNA and ranslate the RNA into immunoglobulin proteins. Moreover, out of the large repetoire of immunoglobulin genes, each antibody-producing cell generally expresses only one light chain gene and one heavy chain gene. It is of fundamental importance to the study of the immune system, and to the study of gene regulation during development, to learn why only certain cells express immunoglobulin genes, and how these cells are able to transcribe only two of them while leaving many similar genes inactive.

Our approach to this problem is to focus on one particular immunoglobulin gene, cloned from the mouse, that synthesizes a kappa light chain. This gene has been inserted on a plasmid that also contains a large part of the animal virus polyoma, allowing it to replicate in mouse cells. For one set of experiments, a second gene was inserted on the same plasmid. This gene was unrelated to the immunoglobulin system and, therefore, should be expressed at equal levels in B-lymphoid and non-lymphoid cells. The final plasmid, designated pLX31, was transfected into two standard non-lymphoid lines of mouse cells, 3T3 and L, and one antibody-secreting lymphoid line, MPC 11. As determined by an S1 nuclease assay of extracted RNA, the second gene was transcribed at approximately equal levels in all three lines of cells, as expected. This proves that the plasmid pLX31 was able to penetrate all of the cell lines in a transcribable state. However, the kappa immunoglobulin gene on the plasmid was only transcribed in the lymphoid MPC 11 cells, with no detectable transcription in the non-lymphoid 3T3 and L cells. Most recently, the same plasmid has been transfected into T-lymphoid cells, with results intermediate between the myeloma cells and non-lymphoid cells.

Two conclusions relevant to gene regulation during development can be inferred from this cell-type specific expression of a transfected kappa gene. First, since the DNA template is in the form of an unintegrated plasmid, the level of

kappa gene activity is not controlled by chromosomal location or large-scale chromosome structure. Second, since the kappa gene was transfected into terminally differentiated cells, activation or inactivation of this gene does not depend on modifications made to it during the process of cell development. Rather, the already differentiated cells contain all the information needed to appropriately regulate the expression of new copies of the kappa gene.

In previous experiments, we have shown that transcription of an immunoglobulin gene requires two DNA elements: (1) the promoter itself and (2) a downstream regulatory element called an enhancer that stimulates transcription from the promoter. In light of the results described above, the question arises whether it is the promoter or the enhancer that confers cell-type specificity on the immunoglobulin gene. Our recent experiments show that in fact both the promoter and enhancer are cell-type specific, i.e., function only in lymphoid cells. To show this, we first placed the immunoglobulin enhancer on a plasmid next to a viral promoter that can function in any cell type. We observed that the enhancer stimulated transcription from the promoter only when the plasmid was transfected into lymphoid cells, indicating that the enhancer is cell-type specific. Conversely, we placed an enhancer that can function in any cell type on a plasmid next to the immunoglobulin promoter. After transfection of the plasmid, the promoter functioned to initiate transcription in lymphoid but not non-lymphoid cells. As the enhancer works in either cell type, this shows that the promoter itself only functions in lymphoid cells.

Significance to Biomedical Research and the Program of the Institute:

The immunoglobulin gene family produces the proteins that constitute antibodies, a crucial aspect of the body's defense against infectious diseases and probably cancer. Understanding how these genes are regulated, especially how they are activated, is therefore of great potential utility in controlling disease processes.

Future Course of Research:

We will attempt to locate more precisely, at the nucleotide level, the DNA sequences involved in regulating expression of immunoglobulin genes. We will also use the differential expression of transfected immunoglobulin genes in lymphoid and non-lymphoid cells to develop an assay for the protein factors that activate transcription of immunoglobulin genes.

Publications:

Queen, C., and Baltimore, D.: An immunoglobulin gene is activated by downstream sequences. Cell 33: 741-747, 1983.

Stafford, J., and Queen, C.: Cell-type specific expression of a transfected immunoglobulin gene. Nature 306: 77-79, 1983.

Queen, C., and Stafford, J.: Fine mapping of an immunoglobulin gene activator. Mol. Cell Biol. 4: 1042-1049, 1984.

Queen, C.: Regulation of immunoglobulin transcription. Oxford Surveys on Eucaryotic Genes 1: 169-191, 1984.

Queen, C., and Stafford, J.: Fine-mapping of an immunoglobulin gene activator. Mol. Cell. Biol. 4: 1042-1049, 1984.

Korn, L.J., and Queen, C.: Analysis of biological sequences on small computers. DNA 3: 421-436, 1984.

Foster, J., Stafford, J., and Queen, C.: An immunoglobulin gene promoter displays cell-type specificity independently of the enhancer. Nature 315: 423, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05267-01 LB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanisms of Plasmid Maintenance

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. Yarmolinsky Chief, Developmental Biochemistry and Genetics Section, LB, NCI

E. Hanson Visiting Fellow LB NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry, DCBC

SECTION
Developmental Biochemistry and Genetics

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to understand mechanisms by which unit-copy bacterial plasmids are stably inherited, we seek to identify and characterize functions of the host that are directly implicated in the maintenance processes. In the period of this report our recently established laboratory has succeeded in resolving contradictory evidence concerning the participation of the dnaA protein of Escherichia coli in maintaining the 90 kb P1 plasmid. It has previously been found that a well-characterized plasmid replicon isolated from P1 carries two copies of a dnaA protein binding site, but appears unaffected in its replication by a mutant allele of dnaA that does not satisfy the dnaA requirement of the E. coli origin of DNA replication. We report here that this P1 replicon cannot do without dnaA protein entirely. On the other hand, a presumably different P1 plasmid replicon, which remains to be isolated and characterized, can integratively suppress a null mutation in the dnaA gene. We suggest that P1 carries both dnaA-dependent and dnaA-independent plasmid replicons. The biological significance of the presence of replicons with differing host-function requirements in so simple an organism as P1 remains to be determined.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

We aim to elucidate mechanisms that achieve DNA replication control and replicon equipartition at cell division. Unit copy bacterial plasmids that are particularly amenable to detailed genetic analysis have been chosen for study. Currently, our investigations are limited to an attempt to resolve contradictory evidence concerning the involvement of the bacterial dnaA gene in the initiation of stringently controlled plasmid replication in prophage P1 and in other similarly organized plasmids. The dnaA gene product is known to be essential for Escherichia coli DNA replication and recently has been implicated in a variety of regulatory circuits.

Methods Employed:

Analysis of the dnaA requirement of various plasmid replicons (several of them cloned in λ vectors) was studied by in vivo methods in bacteria that replicate from an inserted unusual origin. An E. coli completely independent of dnaA function for survival (although not for normal growth) was derived from a strain driven by a dnaA-independent mini-R1 replicon and rendered dnaA-defective by transposon mutagenesis; a mutant with improved growth characteristics (the mutation residing in neither dnaA nor the mini-R1) was routinely used. The presence of appropriate insertions and deletions was verified by Southern blot analyses of DNA restriction fragments.

Major Findings:

Introduction

Highly conserved short DNA sequences imbedded in regions of nonhomology are indicative of functionally important recognition sites. The presence of binding sites for the bacterial dnaA replication-initiation protein within a variety of prokaryotic origin regions suggests that this protein is an essential participant in the replication of a number of plasmids as in the replication of the bacteria that are host to them. Paradoxically, plasmids F and P1 and certain other plasmids endowed with dnaA protein binding sites, but otherwise nonhomologous, have been taken to be independent of dnaA because, if chromosomally integrated, they can support the replication of dnaAts bacteria at "non-permissive" temperatures. Evidence for the presence in P1 (and F) of more than one replicon potentially capable of this integrative suppression suggested to us the possibility that a basic replicon of P1 that bears dnaA binding sites might not be capable, in isolation, of integrative suppression. However, direct tests of such an isolated replicon proved it to be an entirely adequate effector of integrative suppression of dnaAts mutations. The paradox remained.

Recent evidence for residual dnaA activity in dnaAts E. coli mutants incubated at 42° (from studies of lethality suppression by secondary mutations in topI) reopens the question of whether plasmids capable of integrative suppression are entirely independent of dnaA function. Moreover, definitive tests of dnaA-independence are currently possible: null mutants of dnaA (insertions and internal deletions that do not interfere with the functioning of the dnaN gene,

a downstream member of the dnaA transcription unit) have become available. M. Yarmolinsky observed that transposon inactivation of dnaA in a rnh host, which must replicate from abnormal origins, appeared to block transformation by a mini-P1. This preliminary result has been the stimulus for a thorough reexamination by Egon Hansen of the effect of dnaA null mutations on the plasmid replication of P1 and a basic replicon derived from P1.

Results

I. Integrated P1, But Not Similarly Integrated λ-mini P1, Can Integratively Suppress a dnaA Null Mutation

E. coli strains harboring P1 or λ-mini P1 at the identical chromosite were constructed by prophage displacement. Chromosomal P1, but not chromosomal λ-mini P1, permitted introduction of a dnaA::Tn10 mutation. The positive finding indicates a capacity of intact P1 to replicate as an oversized plasmid in the absence of dnaA function; the less conclusive negative finding is suggestive of an absolute requirement for dnaA in plasmid replication of the mini-P1, although this requirement might be specifically associated with the size of the attached DNA.

II. Free P1, But Not λ-mini P1, Can Be Established As A Plasmid in the Complete Absence of dnaA Function

The capacity for plasmid replication of P1 and of att-deleted λ vectors with replicon inserts derived from P1, F and col E1 was tested in a dnaA::Tn10 host. Of these replicons only the λ-mini P1 and λ-mini F failed to lysogenize as plasmids. These results suggest that P1 and F possess both dnaA-requiring and dnaA-dependent plasmid replicons, although the results might possibly be interpreted as a specific failure of the mini-plasmids to establish, but not maintain themselves as plasmids.

III. Established λ-mini P1 Plasmids Are Lost Upon Removal of the dnaA Gene

A dnaA::Tn10 (λ $dnaA^{+}$) merodiploid in which a heteroimmune λ-mini P1 had been established was selectively cured of the prophage bearing the intact dnaA gene and invariably found to lose the mini-P1 plasmid concomitantly unless the chromosomal dnaA allele had become wild type by recombination. The same result was obtained with a high-copy number (ΔincA) mutant of λ-mini P1, whereas loss of the wild type dnaA gene did not affect pBR322 stability. Plasmids pBR322 and λ-mini P1ΔincA are of comparable copy number. These results strongly support the conclusion that the mini-P1 replicon (but not pBR322) is absolutely dependent upon dnaA function.

IV. Lethal Overreplication of the Bacterial Chromosome From An Integrated Mini-P1 Origin is dnaA-dependent

Recent results of Subrata Pal and Dhruba Chattoraj indicate that an origin region from mini-P1, when chromosomally integrated, can be lethal to the bacterium if an appropriate excess of P1 replication protein (gp repA) is furnished from a cloned repA gene. In E. coli harboring chromosomal mini-R1 and the mini-P1 origin, we find that no amount of RepA protein induces lethal

replication when a dnaA deletion has also been introduced. The requirement for both DnaA protein and RepA protein to obtain lethal initiations of replication provides further support for the dnaA dependence of mini-P1. Moreover, it indicates that this dependence is due to a more direct role of dnaA than that of regulating repA expression.

Significance to Biomedical Research and the Program of the Institute:

The present studies advance knowledge of how DNA synthesis is controlled in a class of well-defined replicons. They suggest clues to the understanding of comparable regulatory circuits in the cells of higher organisms and, by analogy with other advances in this area of basic research, are likely to affect biotechnology in ways that are no less important for being entirely unpredictable.

Future Course of Research:

We will attempt to define more clearly the replicon(s) of P1 that are not dependent upon dnaA, and compare it (them) with the dnaA-dependent replicon that we have characterized in some detail. The planned enlargement of the section and the opportunities for collaboration with members of other NIH laboratories will permit us to undertake a broader study of the involvement of host functions in plasmid maintenance as well as more detailed studies of mini-P1 replication and its remarkably precise control. We look forward to initiating studies of other functions that connect plasmid maintenance to the control of cell division.

Publications:

Yarmolinsky, M.: Bacteriophage P1. In Laskin, A.I., et al. (Eds.): CRC Handbook of Microbiology. Boca Raton, FL, CRC Press (in press).

LABORATORY OF MATHEMATICAL BIOLOGY

SUMMARY

October 1, 1984 through September 30, 1985

The activities of the Laboratory of Mathematical Biology (LTB) fall into several broad areas: macromolecular structure and function, membrane structure and function, immunology, pharmacokinetics, and computational and modeling methodology. The work is both theoretical and experimental. Application of theoretical understanding to these biological systems, which serve as models for aspects of the cancer process, is accomplished through the use of advanced computing. Close collaboration provides valuable feedback and knowledge transfer between these two research domains. The Laboratory consequently develops computer methodology that is utilized by researchers of the entire biomedical community.

Application of Sequences Analysis to Structure of Virus and Cell Proteins. In the Office of the Chief computerized analyses are used extensively along with techniques of biochemistry, virology, and electron microscopy to study picornaviruses, adenoviruses and other virus-cell systems. Sequences of picornaviruses, typified by polio, rhino, hepatitis, and foot-and-mouth disease, are examined for relationships within the family and to other known and hypothetical proteins. Secondary structures of the RNAs have been found to vary with respect to pathological and sequence variances. A single base change at position 472 in Sabin polio type 3 correlates within reversion to neurovirulence. It also changes the predicted stem and loop secondary structure (J.V. Maizel, K. Currey and J. Owens). Adenoviruses are studied with a goal to understanding early events in virus replication wherein the cell's metabolism is subverted to viral functions, and late events during which assembly and morphogenesis occurs. Early viral proteins, whose existence is known from biochemical studies, are analyzed by comparing their sequences to cellular proteins of known function. Physical association between the viral E3 glycoprotein and cellular MHC proteins correlates with homology between their sequences. Reovirus protein sigma-1 was found to have a heptapeptide pattern typical of a coiled-coil, alpha-helical structure (D. Chatterjee, J.V. Maizel, and J. Owens).

New analyses of proteins and nucleic acids are developed and implemented. Graphic representations revealing homology, and reverse complementarity are coupled with numerical methods to aid the prediction of secondary structure, splicing, promoters, and recombination in nucleic acid molecules. Programs are developed and installed in VAX systems designed for sequence analysis. Structures of up to 2000 bases are predicted. Methods to assess the significance of predictions use Monte Carlo simulations, evolutionary comparisons and biochemical data. Protein secondary structure is predicted from amino acid sequences. New sequences are compared with computerized databases to detect relationships with known proteins. Planning and procurement is underway for the development of a close interaction with an advanced supercomputing facility at the Frederick Cancer Research Facility. This machine will be the first

scientific computer dedicated entirely to biomedical research (R. Nussinov, J.V. Maizel and J. Owens).

Theoretical Molecular Structure. In the laboratory we are studying biological macromolecules and their properties. Both long range and short range interactions in proteins have been investigated by tabulating data from X-ray crystal structures. The long range study has included solvent interactions by counting the numbers of other residues around each type of amino acid; if this shell of residues is incomplete it is assumed to be filled with a number of equivalent groups of water molecules. On this basis, we have obtained effective interaction energies between all types of pairs of amino acids (S. Miyazawa and R. Jernigan). We are applying these energies to locate hydrophobic nuclei and attempting to incorporate them into a protein folding scheme. Molecular modeling has been proceeding in three areas: membrane channels composed of amphipathic helices, DNA local conformations and DNA-protein interactions. For the membrane proteins this model construction proceeds by combining experimental data with calculations of preferred locations and orientations of helices with respect to membrane boundaries, helix-helix packing, formation of charge pairs and disulfide bonds (H.R. Guy and P. Seetharamulu). Proteins that have been modelled are acetylcholine receptor, interleukin 2, δ hemolysin, α toxin and action potential sodium channel. Local sequence dependent DNA conformations have been proposed (A. Sarai, R. Nussinov and R.L. Jernigan). These results include both smooth and sharp bends. For the DNA-protein interactions, we evaluate the strengths of various types of DNA-protein and place the repressor in the most favorable orientation within the major groove of the operator region. Subsequent comparisons are made with experimental binding constants of various operators and their mutants; we investigate the uniqueness of the binding to the operator, compared to the entire lambda DNA (A. Sarai and R. Jernigan). Computer color graphics systems assist in these modeling efforts (K. Ting, R. Jernigan, P. Seetharamulu and S. Miyazawa). The effects of drugs and carcinogens on the electronic structure of DNA are calculated (G. Barnett). The B form to Z form transition in DNA has been studied with a simple mechanical model and a detailed statistical mechanistic model; comparisons with experiments on plasmid DNA with inserted regions of (G-C) were made (A. Sarai, R. Jernigan and S. Miyazawa).

Simulation, Analysis and Modelling of Physiological Systems. General purpose computer programs (SAAM, CONSAM) for the simulation of compartmental models of bio-kinetic systems which may be used by investigators not sophisticated in mathematics or computer programs are developed. This development was initiated in 1959, and the program continues to be expanded and revised as new features are added, and old features are re-examined. Along with the development of computer tools to test compartmental models against a relational data-base through a specific sub-language grammar.

Through interactions with Dr. R. Boston of LaTrobe University, Australia, their has been continued development of the computer system (SAAM) for the simulation, analysis and modeling of bio-kinetic systems. Further development of a conversational mode (CONSAM) of operation increased the versatility, applications and automated the modeling process. The programs which make up SAAM have been revised so that they will execute on IBM 43XX series of computer. In addition it has been tested on a new series of dec computers, MicroVAX I and is 35% as productive as the older VAX 11/780. This is the first microcomputer capable of

executing all the programs which make up SAAM. These additions make the SAAM program available to a wider range of users, including the individual user with a microcomputer.

The ability to test the SAAM (CONSAM) programs and there interactions in the user environment has been developed which will facilitate the implementation of a self testing by comparison with predetermined, correct responses.

Application of the SAAM programs for the metabolism of chylomicrons in rats - using this model it was determined that the immediate nutritional status of the rat has a major effect on the mechanism of triglyceride metabolism. Hydrolysis of chylomicron triglyceride is elevated in the standard state as compared to increased uptake of chylomicrons as intact lipoproteins in the chow-fed animal. These studies lead to the prediction that hydrolysis of triglycerides will be decreased in the low fat nutritional state recently associated with cancer prevention.

Further analysis of lipoprotein metabolism indicates that the apoB/E receptor plays a major role in apoB-100 metabolism but does not affect the apoB-48 metabolism. In addition the apoE phenotype in humans is a determinant in the kinetics of low density lipoprotein metabolism through the apoB/E receptor.

Modeling of the endocrine system has continued on lipoproteins, the glucose-insulin system and receptors.

Detailed modeling of ketone bodies has also been carried out (M. Wastney) in collaboration with Dr. S. Hall of Ottawa, who carried out the experiments. This relates to other modeling carried out by this group on intermediate metabolism. Studies on insulin secretion in various populations and its variation as a function of glucose load have also been studied.

Membrane Structure and Function. The studies in the Membrane Structure and Function Section (R. Blumenthal, A. Walter, O. Eidelman and M. Ollivon) deal with the the insertion and organization of molecules (proteins, lipids) in membranes, interactions of membranes with toxins, viruses, channel proteins and polypeptides, and membrane fusion. Spectroscopic techniques (fluorescence, circular dichroism) are used to study lipid-protein interactions and membrane fusion, and conductance across planar black lipid membranes (BLMs) is measured to approach questions of membrane destabilization and channel formation.

Studies on membrane fusion focus on reconstitution of viral spike glycoproteins into lipid vesicles and on membrane fusion mediated by the resulting virosomes. A rational basis for the reconstitution process is being developed by following dissolution by detergent, and reassembly of phospholipid and protein using fluorescence energy transfer techniques. Factors that control fusion (e.g. pH, surface charge, lipid headgroup, vesicle size, protein conformation) are examined in order to elucidate the fusion mechanisms. A theoretical framework is being developed to model aggregation and fusion kinetics (D. Covell, P. Greif). Efforts are directed at constructing virosomes which fuse with cells in order to deliver materials to the target cell membrane or cytoplasm.

Studies on channel-formation with cytolysin isolated from large granular lymphocyte tumors and from cytotoxic T lymphocytes are being pursued with lipid vesicles and planar bilayers to examine the mechanism of its lytic action. Recently it has been predicted that Interleukin-2 (IL-2) might form channels in bilayers (H.R. Guy). Experiments using planar bilayers and phospholipid vesicles containing voltage-sensitive dyes show pH-dependent and voltage-dependent permeability changes induced by this molecule. This finding might have important implications for the biological action of IL-2. In order to elucidate the mechanism by which adenovirus gains access to the cytoplasm of the host cell, via receptor-mediated endocytosis, its interaction with lipid membranes has been studied. The results indicate a pH-dependent permeability change in the liposomes, which parallels the pH-dependence of the disruption of endocytic vesicles by adenovirus.

The research group of J. Weinstein studies the delivery of monoclonal antibodies via lymphatic vessels for diagnosis and possible treatment of lymph node metastases (D. Covell, O.D. Holton, C.D. Black, M.J. Talley, J. Barbet and J. Weinstein). To establish a firm pharmacologic basis in pre-clinical studies, antibodies to normal cell types in the lymph node were studies initially. Antitumor antibody was then successfully delivered from subcutaneous injection sites to lymph node micrometastases of an animal tumor. Parallel studies are being done with antibody-toxin conjugates and antibody-alpha emitter conjugates for possible therapy of tumor in lymph nodes and for modulation of the immune response to tumors. Experimental results are used to construct pharmacokinetic models (using the SAAM system). The animal studies extend directly into clinical protocols for detection of melanoma, lymphoma, breast carcinoma, and non-small cell lung carcinoma in lymph nodes. The clinical protocol on lymphoma has produced the most efficient imaging of tumor cells yet achieved in humans by any non-invasive technique.

For tumor cells far from the nearest lymphatic or blood vessel, binding of an antibody (or other ligand) may be limited by the rate at which the molecules can "percolate" through the extracellular space. Spatial and temporal profiles of immunoglobulin distribution generated by diffusion and convection through tumors are being studied using a program package for solution of partial differential equations. The analysis takes into account specific binding, nonspecific binding, and metabolism. One surprising prediction is that low affinity antibodies will sometimes be more effective in therapy than higher affinity ones. The theoretical predictions are being tested experimentally in micrometastases and spheroids.

Theoretical Immunology. The programs of the Theoretical Immunology Section (C. DeLisi) focus primarily on (1) the development of physical chemical principles and their integration with artificial intelligence techniques, with the goal of predicting the function, location and structure of proteins; (2) applications to various problems in molecular biology, with an emphasis on molecular immunology. Much of the research is carried out in close collaboration with experimental scientists at the NIH and throughout the Nation. Among the current and anticipated projects are (1) the development of new techniques for predicting the tertiary structure of immunoglobulin family molecules with applications to cell interactions; (2) the prediction of sites recognized by antibodies and T cell receptors and the development of vaccines based on such prediction (H.

Margolit); (3) the development and application of secondary structure predictive methods, based on both statistical and physical chemical information, that would be accurate and widely applicable (S. Le and J. Cornette); (4) the development of physical chemical principles governing macromolecular stability including application and development of quantum chemical techniques to study charge distributions on interacting residues in different environments (G. Barnett), and the role of solvent in determining the free energy of interaction between various types of side chains (J. Spouge); (5) development and application of pattern recognition algorithms (P. Greif) to identify various sites on nucleic acids such as exon/intron boundaries (M. Kanehisa and K. Nakata).

Although the thrust of the section is predominantly molecular, collaborative efforts continue with a number of intramural laboratories on cellular and systemic level modelling. These currently focus on the development and application of methods for analyzing complex systems such as those encountered in the feedback loops that regulate responsiveness, tolerance and memory (J. Eisenfeld), and in cellular level phenomena such as endocytosis and down regulation (M. Gex-Fabry.).

Image Analysis. Work in the Image Processing Section (L. Lipkin) studies 2D Gel analysis (P. Lemkin), nucleic acid secondary structure (B. Shapiro) and the necessary hardware and software systems support (M. Schultz). As part of the development of the GELLAB system, a set of detailed specifications have been developed which define a minimal cost system, appropriate to a small university biochemistry laboratory. In this regard work has continued on the conversion of the GELLAB software from SAIL to C has progressed significantly with the major part of the PSAIL translator completed. Collabortions with Dr. Sonderegger, University of Zurich (Switzerland), is proving most productive as part of the collaboration involving the proteins of neural axonal synthesis and computer identification of possible coordinated precursor product pairs. Collaboration is also continuing with Dr. Lester of University of Tenn. on the Leukemia 2D gel data base.

Research continues in collaboration with Dr. R. Nussinov from the School of Medicine at Tel Aviv University in the area of B-DNA distortions based upon the Calladine-Dickerson rules. Two extendable systems have been developed and enhanced which incorporate the four rules of distortion, namely, helical twist, base pair roll, torsion and propeller twist. These systems have the facility to search for patterns that are not known to exist a priori. Feature enhancement and feature detection techniques are available to aide the user in an interactive environment to discover new patterns of potential interest. These techniques have indicated the existence of morphologic structures which appear to be correlated to functionality within the molecule. Work has also begun on a new algorithm to measure similarity among secondary structures of RNA molecules. This technique when perfected should permit the determination of multiple levels of similarity among several molecules of the same or different classes. RNA secondary structure drawing programs have been sent to several more institutions around the world. It is proving to be quite useful in depicting the structures of RNA molecules after they have been folded.

Work on system software has been suspended following completion of a working package for image file transfer, Spider, as has further development of the intralaboratory computer network.

Membrane Biology. The Membrane Biology Section continued research, development and application of new cytochemical techniques ("fracture-label, label-fracture, fracture permeation") for high resolution localization of molecular components of plasma and intracellular membranes as well as the cytoplasm, nucleoplasm and extracellular matrices. Investigation of the mechanisms of assembly of intercellular junctions and the processes of membrane fusion and reorganization during the acrosome reaction in sperm cells is pursued.

Fracture-label is used to study localization of transmembrane proteins in sperm plasma membrane and of glycoconjugates in acrosome membranes, localization of glycoproteins within the nuclear matrix, localization of butyrophilin in mammary gland epithelial cells, and localization of cholera toxin receptors in human neutrophils (A. Aguas, M.L. Barbosa, F. Kan, I. Mather and P. Pinto da Silva).

Label-fracture is developed as a technique for high resolution mapping of surface microdomains on sperm cells cytochemistry of the apical membrane of toad bladder epithelial cells and capping of surface IgG in B lymphocytes (F. Kan, M.L. Barbosa, J. Chevalier and P. Pinto da Silva).

Fracture-permeation is developed for study of intermolecular spaces in model gels, intermolecular spaces in the cytoplasm, changes in compactness of the cytoplasm related to cellular activation, development, muscle contraction. Compactness of extracellular matrices in rat and human glomeruli is studied (M.L. Barbosa, J. Bariety's group and P. Pinto da Silva).

Intercellular junctions/membrane fusion assembly of tight junction strands at the basal pole of epithelial cells is studied in response to osmotic reversal, and membrane fusion and dynamics during the acrosome reaction in boar spermatozoa (J. Chevalier, A. Aguas and P. Pinto da Silva).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08300-13 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: SAAM, Development and Applications

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Loren A. Zech, M.D., Senior Investigator, LTB, NCI
Detail from OD, NIHLB

COOPERATING UNITS *(if any)*: Dr. Ray Boston, LaTrobe Univ., Australia; Dr. Naomi Sager, New York, Univ., NY; Dr. Trevor Redgrave, Boston Univ.; Dr. Charles Schwartz, Medical Coll. of Virginia, Richmond, VA; Dr. Waldo Fisher & Dr. Bruce Patterson, Univ. of Fla., Gainesville, FL

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Continuing development of a computer system (SAAM) for the simulation, analysis, and modeling of bio-kinetic systems. Development of a library of benchmark problems were extended to include examples which can be used to verify the implementation of newer integration methodology into the program. Performance studies with SAAM and CONSAM on the Micro VAX I indicates that the micro VAX has about 35% of the performance of the VAX 11/780 for a single user and that system performance while running SAAM and CONSAM is highly dependent on disk space management, the neglect of which can result in a 30 fold degradation in performance. Further development of the conversational mode of operation was achieved through the structuring of comments into groups which allows the testing of a maximum number of processes with a minimum number of comparisons to known responses.

Applications of the SAAM program in the development of compartmental models for the metabolism of chylomerions (triglyceride rich lipoproteins secreted from the intestine) were used and show that a single meal 4 hours before the turnover of chylomicron results in a large change in the delipidation index compared to fasting animals and predicts that thie change will be considered in a cancer prevention low-fat diet.

The development of a compartmental model for the metabolism of vanadium in sheep resulted in the prediction that intravenous route of administration results in a decreased percentage of uptake by the bone and plasma binding proteins and increased rapid excretion in the urine when compared to the oral route of administration.

Further analysis of lipoprotein metabolism indicates that the fractional catabolism of VLDL apoB-48 and apoB-100 and plasma apoE are decreased and that apoE synthesis was less than 1% compared to normals in the syndrome of apoE deficiency.

Cooperating Units (Continued):

Dr. Barbara Howard, Phoenix Research Station, Phoenix, AZ; Dr. H. Bryan Brewer, Dr. Richard E. Gregg, Dr. Carlo, Gabelli, Dr. Dubo Bojanovski, Dr. James Osbourne, Molecular Diseases Branch, NIHLB; Dr. Y-Da Chen, & Dr. Gerald Reaven, Stanford Univ., Stanford, CA; Dr. Kathy Holzsome Cancer Prevention Studies Branch, NCI; Dr. Orville Lavender, Beltsville Human Nutrition Research Center, USDA; Dr. Blossom Patterson, Operations Research Branch, NCI.

Project Description

Project #1 The development of mathematical and computer tools for the simulation of and analysis of Bio-kinetic data and the implementation of these tools within the framework of SAAM and CONSAM.

Objectives:

The development of a general purpose computer program for the simulation of compartmental models of bio-kinetic systems which may be used by investigators not sophisticated in mathematics or computer programs. This development was initiated in 1959, and the program continues to be expanded and revised as new features are added, and old features are reexamined. The development of computer tools to test compartmental models against a relational data-base through a specific sub-language grammar.

Major Findings:

1) Through interactions with Dr. Ray Boston of La Trobe University, Australia, two additional numerical integration techniques which were recently included as a part of the SAAM software were examined in detail and compared to the previously used numerical techniques. Small but significant increases in the economy of simulation were found for the new methods of integration however only for a class of special problems. It was further determined that if the newer methods were case in a more specific framework that a wider class of systems of differential equations could be solved. A previous set of bench mark problems was extended to include problems which can be used to verify the implementation of all integration methods.

The commands in CONSAM were grouped into eight groups to facilitate validation of the response from each command while only testing the response to a limited number of commands. Software was implemented for the validation of every copy of CONSAM with the anticipation of providing a self-testing feature in future versions of CONSAM.

Details of a methodology for increasing the number of data points which can be simultaneously examined using SAAM was developed. This methodology will be further evaluated and then implemented through future interactions wich the group at La Trobe University

Further studies were undertaken in collaboration with Dr. Boston of La Trobe University resulting in the measurement of performance results of the MicroVAX-I. The results relating to SAAM & CONSAM performance on the MicroVAX-I composes process times, accuracy and reliability of the same program on a VAX 11/780. The investigation was constrained as follows: MicroVAX-I with 2 megabytes of physical memory, 31 megabytes of secondary memory, MicroVMS 1.0 operating system, SAAM and CONSAM compiled under VMS 4.0 and a VAX 11/780 with 4 megabytes of physical memory, 451 megabytes of secondary memory, VMS 3.7 operating system and SAAM and CONSAM compiled under VMS 3.7. Major findings indicate that the MicroVAX-I is equal to about 35% of VAX 11/780 for a single user, a major degradation of the MicroVAX-I performance up to 30 fold can result from failure to manage disk space, code sharability does not appear to enhance the processor power of the MicroVAX-I substantially, and several problems were found in the FORTRAN 4.0 compiler.

2) Development of a relational, linguistic, structured-text data-base using the method of sub-language analysis to provide data on metabolic systems organized to facilitate the development and testing of models was continued in collaboration with Dr. Naomi Sager from the computer linguistic group at New York University. In the past year development of an intermediate representation to serve as a bridge between the unstructured free-text input and the structured-text representation. This resulted from the development of a partial sublanguage grammar. The adequacy of the sublanguage grammar was tested by searching a small pool of test papers for specific relationships, and found to be underdeveloped for generalized usage in the field of lipoproteins.

Project Description:

Project #2 Application of SAAM and CONSAM to the Simulation and Analysis of Bio-kinetic Data Analysis.

Objectives:

To develop general purpose mathematical models for particular physiologic system which will lead to the understanding of the kinetic-dynamic behavior of the systems. After the models are developed they are then used to separate out specific parameters of the systems and through drug and diet modulation of the physiologic system obtain a relationship between the kinetic parameters and the dynamic behavior of the system under study. While this is not always the case, many of the models are developed to further understand the response of a physiologic system to an injection of a tracer substance. These objectives have been met through the study of the following systems listed in this report and others for the Laboratory of Mathematical Biology.

Further the analysis of data from particular physiologic systems provides a window through which the ongoing need of the users of SAAM and CONSAM can be assessed. This assessment is then combined with mathematical techniques to become the starting point for further development in project #1 above.

Methods Employed:

When turnover data first becomes available, preliminary analysis consists of fitting the plasma decay curve with a mathematical function, usually a sum exponentials, and determining the area under the decay curve. After extrapolation of the curve back to zero time, the volume of distribution for the isotopic tracer is calculated from dilution and checked for consistency against an independent estimate of the patient's volume. If the difference between the estimate of volume of distribution is small, the area under the decay curve is used to calculate residence times for the radiolabeled apolipoproteins before constructing a compartmental model. If, however, the difference is large, calculations of the residence time is not completed until sufficient data has been collected and a compartmental model has been constructed.

The methods used for the development of multicompartmental models using turnover data from radiolabeled apolipoproteins and plasma lipoprotein studies have detailed in previous reports. These models are simulated using the SAAM simulator (a large collection of digital computer programs run on the Digital VAX-11/780 computer in the Laboratory of Mathematical Biology, National Cancer Institute. These simulated results are compared to the experimental results and the connectivity (number and topology of compartments) as well as the flow of tracer or tracee in the model changed until a working model is developed. Using the model, the volume of distribution of the apolipoproteins is estimated and compared to independent estimates of these volumes. After development of the

compartmental model, the parameters of the model are adjusted using nonlinear least squares techniques resulting in minimal least square error. These have now been extended to studies in which differences in multicompartmental models, developed using turnover data from two separate radiolabels on two different apolipoproteins in normal individuals, have been compared.

Major Findings:

1) In conjunction with Dr. Trevar Redgrave of Boston University, Laboratory of Biophysics, a compartmental model for the plasma and liver metabolism of chylomicrons was used to examine the effect of the nutritional status of the recipient physiology system on metabolism. Of major interest was the action of lipase on the hydrolysis of chylomicron triglyceride. Because previous findings have shown two major features contributed to the kinetics of chylomicron triglyceride metabolism an "index of delipidation" was developed which was only sensitive to the contribution of hydrolysis to chylomicrons triglyceride kinetics. Significant differences in the delipidation index were found between groups of recipients who were fasting and those who received a standard meal. Hydrolysis was much faster in the fasting group. This increase in efficiency of hydrolysis must now be examined in the low fat meal conditions.

2) In collaboration with Dr. Waldo Fisher and Dr. Bruce Patterson of the University of Florida a kinetic model for vanadium metabolism was developed. This model is consistent with data obtained from sheep fed a control diet containing 2.6 ppm vanadium or 200 ppm supplemental vanadium. Sheep were administered ^{48}V dioxvanadium either orally or intravenously and blood, feces, and urine radioactivity were followed for 6 days. New insights included a) a significant absorption of ^{48}V occurs from the gastrointestinal tract, b) an in vitro processes converts ^{48}V dioxovanadium to a more biologically reactive species, c) at steady state the upper and lower gastrointestinal tracts contain 10 to 100 fold more vanadium than the plasma (blood). No statistically significant differences in transport rates were detected between animals receiving 0 or 200 ppm supplemental dietary vanadium.

3) In conjunction with Dr. Ernest Schaefer the kinetics of radiolabeled apoB-100 and apoB-48 were examined in a patient with apoE deficiency. Homozygotes for apoE deficiency had accumulation os apoB-48 and apoA-IV in their lipoproteins in contrast to normals and heterozygotes. Radiolabeled VLDL apoB and apoE kinetic studies show that this subject homozygous for apoE deficiency has decreased fractional catabolism of VLDL apoB-100, apoB-48 and plasma apoE with mean residence times of .93, 1.76, and 1.50 days respectively compared to .12, 0.4 and .73 days respectively in normal controls. ApoE synthesis was less than 1% or normal. These findings are used to predict that homozygous familial apoE deficiency is a cause of tye II hyperliprotemia, is due to a lack of apoE production, and that apoE is essential for the normal catabolism of triglyceride-rich lipoprotein constituents.

4) In collaboration with Drs. Y-D Chen and Gerald Rowen of Stanford University, LDL apoB metabolism in normal, and diabetic rabbits had been examined. Data from these studies when analyzed by determining the area under the curve indicates

that the residence time of LDL apoB does not change when diabetic and control rabbits are compared. Decreases in synthetic rates of LDL apoB result from decreased conversion of VLDL apoB (triglyceride rich lipoprotein apoB) to LDL apoB.

5) In collaboration with Dr. Oscar Lenaris of the University of Michigan a model has been developed for the metabolism of norepinephrine. The previous one compartment model for plasma norepinephrine kinetics has been expanded to include a non vascular exchange compartment resulting in the revision of the previously calculated synthesis rates to a lower value. Experimental and theoretical examination of the norepinephrine are now underway which will further reverse the instrument of synthesis.

6) In conjunction with Dr. Schwartz from the Medical College of Virginia, free cholesterol metabolism has been studied in a patient deficient in apoB-100, the protein responsible for the assembly and secretion of triglyceride rich lipoproteins from the liver and intestine. Absence of the protein in the plasma occurs in the malabsorption of lipids. Because this patient has no VLDL or LDL she was used to test the hypothesis that HDL is the preferred lipoprotein which supplies plasma cholesterol for the formation of bile acids. Near normal secretions of bile acid in this patient indicates that HDL is sufficient for bile acid synthesis. This study will be used to test the present compartmental model for free cholesterol metabolism.

7) In collaboration with Dr. Mark Weaver, NHLBI, a model for the metabolism of α_1-antitrypsin was used to investigate the pharmacokinetics of replacement in deficient subjects. Preliminary examination of the space of distribution resulted in the prediction that patients received a two fold increase in the infusion rate when assigned with the same methodolgy as the plasma concentration.

8) In collaboration with Dr. J. Yarmish of Columbia University, VLDL triglyceride synthesis rates were determined following a constant infusion of ^{14}C labelled free fatty acids in trauma subjects and compared to similar studies in nutrient depleted subjects. Using a multicompartmental triglyceride model it was predicted that no difference in triglyceride synthesis rates was observed, and that triglyceride synthesis rates were up to double those previously measured in depleted subjects.

Significance to Biomedical Research and the Program of the Institute:

Understanding the metabolism of lipoproteins and the moieties which up these lipoproteins (cholesterol, cholesterol ester, triglycerides, and apolipoproteins) are significant because of their relationship to atherosclerosis and cholelithiasis. The development of a concept of plasma lipoprotein metabolism is approached by the theoretical analysis of data from metabolic studies using the techniques of compartmental and statistical model building. This type of theoretical analysis provides a framework for discussion between investigators. This project consists of the testing and further development of compartmental models for lipoprotein metabolism as well as the proposal of new models where they do not exist.

Understanding lipoprotein metabolism is of major importance due to the central role of lipoproteins in the transport and catabolism of cholesterol and triglycerides in normal and patients with disorders of lipid metabolism and/or atherosclerosis. Because of the recent elucidation of the negative correlations between HDL-cholesterol levels and the incidence of coronary heart disease the understanding of the two major HDL apolipoproteins (apoA-I and apoA-II) is particularly relevant to the understanding of atherosclerosis.

Understanding the modulation of lipoprotein, cholesterol, cholesterol ester, triglycerides and apolipoproteins by drugs, diet and genetic disease is also of significance since changes in these effectors may have major effects on atherosclerosis and cholelithiasis. This theoretical analysis also provides a framework for comparison between groups as dissimilar as caucasians and American Indians.

Furthermore, it is intended that the study of the lipoprotein metabolic system serve as a particular system in which to develop the techniques of analysis to large (more than 20 simultaneous differential equations) metabolic systems these findings can later be applied to many different systems.

Proposed Course:

Detailed studies will be continued on the analysis of the differences in apoA-I and apoA-II metabolism by further specifying and defining the current compartmental models, with particular emphasis on analysis of studies in abnormal subjects such as type I and Tangier subjects. Preliminary studies will be continued on the compartmental analysis of apoE and apoC metabolism. The overall objective will be the development of a comprehensive model of human lipoprotein metabolism by the incorporation of this formation into previously proposed Lpb models. The formulation of an overall conceptualization of lipoprotein metabolism will be continued by qualitative and quantitative testing of these conceptions using compartmental model, the relational data-base and, and other theoretical methods. Of particular interest will also be the determination of which parameters are modified by diet, drug and transformed by genetic disease.

In addition to the studies made to examine the influence of radiolabeling apoA-I, the effects of radiolabeling apoB will be examined in more detail. These types of studies are important in that they point out the differences in kinetics which are associated with the tracer methodology and bring to the surface the set of results which are independent of the methodology and hence most representative of physiology.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08303-13 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Membrane Dynamics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

Robert Blumenthal, Ph.D., Chief, Membrane Structure & Function Section, LTB, NCI

Other Professional Personnel:

Anne Walter, Ph.D., Staff Fellow	LTB, NCI
Ofer Eidelman, Ph.D., Visiting Fellow	LTB, NCI
Michel Ollivon, Ph.D., Guest Worker	LTB, NCI
Peter Greif, M.D., Staff Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Pierre Henkart, IB, NCI; Dr. Richard Schlegel, LP, NCI; Dr. Ira Pastan, LMB, NCI
Dr. Steven J. Morris, IRP, NINCDS; Dr. Clifford J. Steer, LBM,
NIAMDDK; Dr. Ira Levin, LCP, NIAMDDK

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Structure & Function Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	4.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We study the organization and changes in organization of membrane components (lipids and proteins), both in the lateral and in the perpendicular direction. (1) We follow the insertion of proteins into a preformed lipid bilayer (either in the form of a planar bilayer or of a lipid vesicle), and study the factors which determine the protein's orientation. We measure permeability properties of lipid membranes to study: (a) mechanisms of ion transport; (b) properties of transport systems isolated from natural cell membranes; (c) mechanisms of cytotoxicity; (d) the effect of the membrane potential on the disposition of membrane proteins. (2) We have developed model systems in which fusion of phospholipid vesicles is induced Ca^{2+}, pH, and/or by such proteins as tubulin, clathrin, apocytochrome c and polylysine. We reconstitute viral spike glycoproteins into lipid bilayers and study the mechanism of pH-dependent membrane fusion mediated by those proteins. We study this fusion process using an assay involving resonance energy transfer between two fluorophores incorporated into the vesicle bilayer. (3) We observe lateral organization and movement of fluorescently - labelled molecules on cell surfaces by fluorescence microscopy. We study the mechanism by which asymmetry is maintained between apical and basolateral surfaces in epithelial cells.

Project Description

Other Professional Personnel (continued)

David Covell, Ph.D., Senior Staff Fellow	LTB, NCI
H. Robert Guy, Ph.D., Expert	LTB, NCI
Arthur Bonner, Summer Aid	LTB, NCI

Objectives:

To study the physical mechanisms of ion transport in reconstituted membranes. To develop lipid bilayers as an assay for transport systems isolated from natural cell membranes. To study mechanisms of cytotoxicity. To study the effect of the membrane potential on the disposition of membrane proteins. To study the role played in cell membranes by the mobility and distribution of cell surface receptors. To study the physiological significance of domains of lipid in membranes. To study factors which constrain the movement of membrane protein and lipids to specific areas of the cell surface. To study the mechanism of membrane fusion. To develop fusogenic liposomes as vehicles for delivery of antitumor drugs into cells.

Methods Employed:

The bilayer membranes are formed from natural membrane extracts or pure lipids in an aperture between two electrolyte solutions. The electrical properties of the membranes are measured before and after application of an activating factor. Lipid vesicles are formed by sonication, reverse-phase evaporation and detergent dialysis. Leakage from vesicles is assayed by measuring the increase in fluorescence as vesicle-encapsulated self-quenched carboxyfluorescein is released into the medium and diluted. Spectroscopic changes upon interaction of proteins with lipid vesicles are studied by fluorometry and circular dichroism. Movement of fluorescently labelled molecules (proteins, lipids, carbohydrates) on cell surfaces is monitored by fluorescence microscopy. Other techniques to measure movement of molecules in membranes are fluorescence polarization, fluorescence energy transfer, and fluorescence stopped-flow kinetics.

Major Findings:

1) In order to develop a rational basis for reconstitution of viral spike glycoproteins, we have followed the dissolution of phosphatidylcholine (PC) vesicles by the detergent octylglucoside and their reassembly. Using fluorescence energy transfer between two lipid probes we have defined three phases the lipid-detergent system goes through as the lipid vesicles are dissolved by octylglucoside: a) bilayer expansion; leakage of contents and lipid exchange take place during this phase. b) a steep rise before the critical micelle concentration, which we believe represents formation of membrane discs. c) complete solubilization into micelles.

2) In order to study assembly spike glycoprotein (G protein) from Vesicular Stomatitis Virus (VSV) into membranes we labeled the protein with FITC. The fluorescence was self-quenched when protein micelles were formed in the absence of detergent and fluorescence intensity increased upon addition of detergent and

formation of monomers in detergent micelles. Having characterized the solubilization pattern for the protein, for the lipid, as well as for the protein:lipid mixture we can now define the critical phase the detergent:lipid:protein system must go through to achieve functional reconstitution.

3) In order to characterize pH-dependent polycation-induced fusion of acidic phospholipid vesicles, we developed a new assay in which energy transfer changes were monitored in asymmetric vesicles, where probes were only present in the inner monolayer of the vesicle membrane. This involved outer monolayer exchange of fluorescent label mediated by the nonspecific phospholipid exchange protein isolated from beef liver.

4) In our studies of polylysine-induced fusion of PC vesicles containing negatively-charged phospholipids we find that there must be sufficient binding sites on the vesicles and sufficient polypeptide to achieve effective aggregation. However an excess of the polypeptide limits fusion by steric and charge repulsion. Thus, for fusion to occur, a delicate balance must be struck between these two effects of polylysine on charged vesicles.

5) We studied polylysine-induced vesicle fusion as a function of vesicle size and of polymer size. We observed less fusion with increasing vesicle size; we relate this finding to membrane destabilization as a requirement for fusion. On the other hand, fusion is quite independent of polymer size with polylysines ranging in size from 4000 to 100,000 MW; we relate this finding to the hypothesis that lipid phase boundaries are not required for fusion.

6) We developed a new way to determine the average size and size distribution of lipid vesicles, biological vesicles and viruses based on HPLC. We find that this has advantages over conventional chromatography in that the speed and precision is markedly increased without loss of resolution.

7) Experiments on Ca^{2+}-dependent release of a water soluble marker from phospholipid vesicles induced by cytoplasmic granules from large rat granular lymphocyte (LGL) tumors have been extended to cytotoxic T lymphocytes (CTL). The CTL granules have a lytic activity which is generally similar to that of LGL granules. Purified cytolysin from LGL granules appeared to induce a voltage-dependent conductance change in planar bilayers.

8) Raman spectroscopy on complexes of clathrin with vesicles made of dipalmitoyl phosphatidylcholine (DPPC) indicate that clathrin induces a substantial acyl chain disorder within the hydrophobic region of the model membranes. Similar increased disorder was found in clathrin-coated vesicles. Since the lipid environments of the coated pit and coated vesicle are not significantly different from uncoated membrane domains, the bilayer disorder induced by the clathrin coat might be one of the factors responsible for membrane invagination and coated vesicle formation.

9) Based on the amphipatic character of its α-helices, Dr H.R. Guy has suggested that Interleukin-2 (IL-2) might form channels in bilayers. [illegible] observed pH-dependent and voltage-dependent conductance changes induced by IL-2 in planar bilayers. Similar results where obtained with phospholipid vesicles containing voltage-sensitive dyes. In accordance with Dr Guy's prediction the permeability

changes were proportional to a higher power of the IL-2 concentration, indicating that a number of molecules have to line up in a cooperative fashion in order to form a channel. This finding might have important implications for the biological action of IL-2.

10) Purified adenovirus induced a pH-dependent release of water soluble fluorescent markers from liposomes. The pH-dependence parallels the disruption of endocytic vesicles by adenovirus. These data support the notion that adenovirus, which enters the host cell by receptor-mediated endocytosis, gains access to the cytoplasm by a subsequent pH-dependent disruption of the membrane of the endocytic vesicle.

Significance for Biomedical Research and the Program of the Institute:

Fusion, mobility, distribution and expression of cell surface components are considered to have important implications for cell transformation and for many aspects of the physiology of normal and tumor cells. The studies on reconstitution of viral proteins and fusion might lead to development of fusogenic liposomes as vehicles for delivery of antitumor drugs into cells.

Proposed Course:

A) The BLM will continue to be used as an assay for toxins, viruses and conductance-inducing materials from cells. The notion of voltage - dependent assembly of proteins in membranes will be further explored with cytolysin isolated from LGL granules and with peptides such as IL-2 predicted to be channel-formers. B) Further studies on the mechanism of membrane fusion will focus on reconstitution of viral proteins and fusion of the resulting virosomes. Questions relating to where the pH-dependence resides (lipid or protein), what the lipid dependence is in composition and size of vesicle, conformational changes in the protein, and whether fusion intermediates can be identified, will be examined. Further efforts will be directed to the construction of virosomes which will fuse with cells in order to deliver materials to the target cell membrane or cytoplasm.

Publications:

Steer, C.J., Bisher, M., Blumenthal, R. and Steven: A.C. Detection of membrane cholesterol by filipin in isolated rat liver coated vesicles is dependent upon removal of the clathrin coat. J. Cell Biol., 98: 315-319, 1984.

Blumenthal, R., Millard, P.J., Henkart, M.P., Reynolds, C.W. and Henkart, P.A.: Liposomes as targets for granule cytolysin from cytotoxic LGL tumors. Proc. Natl. Acad. Sci., (USA) 81: 5551-5555, 1984.

Morris, S.J., Gibson, C.C., Smith, P.D., Greif, P.C., Stirk, C.W., Bradley, D., Haynes, D.H., and Blumenthal, R.: Rapid kinetics of Ca^{2+}-induced fusion of phosphatidylserine/phosphatidylethanol vesicles. J. Biol. Chem., 260: 4122-4127, 1985.

Klausner, R.D., Blumenthal, R., Innerarity, T. and Weinstein, J.N.: The interaction of apolipoprotein A-I with small unilamellar vesicles of L-α-Dipalmitoyl phosphatidylcholine. J. Biol. Chem. 1985, in press.

Blumenthal, R.: Membrane Fusion. Current Topics in Membranes and Transport 1985, in press.

Weinstein, J.N., Blumenthal, R., and Klausner, R.D. Carboxyfluorescein Leakage Assay for Lipoprotein-Liposome Interaction. Methods in Enzymology 1985, in press.

Henkart, P., Henkart, M., Millard, P., Frederikse, P. ,Bluestone, J., Blumenthal R., Yue, C., and Reynolds, C.: The role of cytoplasmic granules in cytotoxicity by large granular lymphocytes and cytotoxic T lymphocytes. In Henkart, P. and Martz P. (Eds): Mechanisms of Cell-mediated Cytotoxicity, II Plenum Press, N.Y., 1985 in press.

Levin, I.W., Vincent, J.S., Blumenthal, R. and Steer, C.J.: Use of vibrational spectroscopy in defining the role of clathrin in coated vesicle formation. Biophys. J. 1985, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08306-13 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Kinetic Modeling of Human Plasma Lipoprotein Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

William Beltz, Ph.D.	IPA	LTB, NCI

COOPERATING UNITS *(if any)*: Dr. Scott Grundy, Center for Human Nutrition and Veterans Administration, Dallas, Texas; Dr. Barbara Howard, NIADDK, NIH, Phoenix, AZ; Dr. Frederick Dunn, Joslin Diabetes Center, Boston, Mass.

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.4	.4	.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Kinetic models of plasma apoproteins, cholesterol and triglyceride are being constructed based on data from experiments in man. The models are used to integrate plasma lipoprotein interactions with enzymes and receptors and to provide a better understanding of plasma lipoprotein synthesis and metabolism in health and disease. The models are particularly useful for the rigorous testing of hypotheses, the design of experiments, and the quantification of the effects of various perturbations.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To develop a qualitative and quantitative understanding of lipoprotein metabolism in man to identify abnormalities and drug effects through modeling of apoprotein, cholesterol, and triglyceride kinetics. Because of the need for diverse extensive data on a variety of patients, collaboration with several experimental groups is maintained.

Methods Employed:

Mathematical modeling is the tool used for integration and analysis of the data. This is performed with the help of the SAAM and CONSAM computer modeling programs.

Major Findings:

Studies of low density lipoprotein (LDL) kinetics were continued in patients with coronary heart disease (Vega, et al). Although the patients studied had normal LDL apolipoprotein B (apoB) and cholesterol levels, those which had high plasma triglyceride (TG) levels had high production rates of LDL and high LDL fractional catabolic rates (FCR's). This phenomenon was not observed in patients with normal TG levels.

A comparison of endogenously and exogenously labeled LDL was carried out in collaboration with the laboratories of Drs. Grundy and Howard. In these studies, the kinetics of radioiodinated LDL apoB were compared with those of LDL apoB in which the label was derived from radioiodinated very low density lipoprotein (VLDL). A mathematical model was formulated (Beltz, et al,) which was consistent with this new data as well as that available in the literature. The model allows determination of total VLDL and LDL apoB transport rates, amount of VLDL apoB converted to LDL and amount of LDL apoB synthesized directly (not followed with the VLDL tracer). Results were obtained for a number of human populations: Caucasians with a wide range of LDL cholesterol levels (Kesaniemi, et al,), nondiabetic, obese Pima Indians (Egusa et al), and nondiabetic Pima Indians with differing levels of obesity (Howard, et al). It was found in all populations that not all VLDL apoB was converted to LDL, nor was all LDL apoB derived from VLDL. This direct production of LDL apoB could be important in the genesis of high LDL cholesterol levels, a risk factor for atherosclerosis. It was further found that Pima Indians had both increased FCR for LDL and decreased fraction of VLDL converted to LDL. These two effects combined to maintain low LDL levels in this population. They may also be related in that the direct removal of VLDL (leading to lower conversion of VLDL to LDL) may be mediated by the same receptor as that responsible for LDL catabolism.

Also in collaboration with Dr. Howard's laboratory, a study was performed to determine the effects of tolazamide treatment on the metabolism of VLDL apoB and TG in type 2 diabetic Pima Indians. In this study, simultaneous injections of tritiated glycerol and radioiodinated VLDL were performed. The disappearance of

labeled VLDL apoB from plasma and the appearance and subsequent disappearance of tritiated VLDL TG were followed for two days. This protocol was performed both before and after treatment. Multicompartmental analysis revealed that the production rate of VLDL TG decreased during therapy while the FCR did not change. Surprisingly, the production rate of VLDL apoB did not change while its FCR increased.

The kinetics of VLDL triglycerides were also examined before and during insulin therapy in type 1 diabetics (Dunn, et al). The study was performed to determine the mechanism of action of insulin therapy's effect on VLDL TG concentration. Triated glycerol was injected, the kinetics of tritiated VLDL TG were followed for 48 hours and the results analyzed using a compartmental model. It was found that therapy decreased VLDL TG transport without changing FCR.

Significance to Biomedical Research and the Program of the Institute:

Modeling is important to identify the abnormalities in metabolism responsible in hyperlipemics, diabetics, and other metabolic disorders, given a very complicated system with multiple interactions. It is hoped that by identifying the mechanisms responsible for abnormalities in lipid metabolism and their relations with intermediate metabolism, appropriate treatment can be more specifically geared to abnormalities. Clinically this is relevant to atherosclerosis and disorders associated with atherosclerosis such as cardiovascular disease, diabetes, cachexia, and other metabolic disorders involving lipids.

Publications:

Kesaniemi, Y.A., Beltz, W.F., and Grundy, S.M.: Comparison of clofibrate and caloric restriction on kinetics of very low density lipoprotein triglycerides. Arteriosclerosis 5: 153-161, 1985.

Vega, G.L., Beltz, W.F. and Grundy, S.M.: Low density lipoprotein metabolism in hypertriglyceridemic and normolipidemic patients with coronary heart disease. J. Lipid Res., 26: 115-126, 1985.

Beltz, W.F., Kesaniemi, Y.A., Howard, B.V. and Grundy, S.M.: Development of an integrated model for analysis of the kinetics of apolipoprotein B in plasma lipoproteins VLDL, IDL and LDL. J. Clin. Invest., in press.

Kesaniemi, Y.A., Beltz, W.F. and Grundy, S.M.: Comparisons of metabolism of apolipoprotein B in normal subjects, obese patients, and patients with coronary heart disease. J. Clin. Invest., in press.

Egusa, G., Beltz, W.F., Grundy, S.M. and Howard, B.V.: The influence of obesity on the metabolism of apolipoprotein B in man. J. Clin. Invest., in press.

Howard, B.V., Egusa, G., Beltz, W.F., Kesaniemi, Y.A. and Grundy, S.M.: Compensatory mechanisms governing the concentration of plasma low density lipoprotein. J. Lipid Res., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08320-10 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Peptide Conformations

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert Jernigan, Ph.D. Theoretical Physical Chemist LTB, NCI

Other Professional Personnel:

Sanzo Miyazawa, Ph.D. Visiting Associate LTB, NCI

Percival D. McCormack, M.D., Ph.D., Senior Staff Fellow LTB, NCI

COOPERATING UNITS *(if any)*

Dr. J. Ferretti, Laboratory of Chemistry, NIHLB; Dr. W.R. Church and Dr. D.N. Fass, Dept. of Hematology, Mayo Clinic, Rochester, Minn.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.6	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Statistically derived Phi-psi maps for each type of residue indicate substantial improvements in X-ray data over previous tabulations.

Position effects in regular secondary regions show strong effects for some types of residues, especially proline, aromatic and polar groups.

A simple dipolar solvent model indicates an asymmetry to electrostatic interactions. Favorable interactions appear to be enhanced and extended to longer range.

Meaningful homologies were demonstrated between ceruloplasmin and two blood clotting proteins, Factor V and Factor VIII.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives:

The aim is to study the conformations available to short peptides and, in a systematic way, develop better secondary prediction methods. Studies of homologous proteins can indicate permissible substitutions of amino acids.

Methods Employed:

The conformational properties of peptides are not sufficiently understood. In some cases, single substitutions of amino acids can frequently cause significant changes in conformation beyond those expected from existing theories; whereas in other cases similar substitutions probably cause no significant change. Based in part on our previous studies of protein secondary structures, we have been pursuing studies on the sequence dependence of conformation in several ways. These have included statistical surveys of X-ray crystal data to investigate the position dependences of amino acids in secondary structures, tabulation of statistical Phi-Psi maps, simulation of solvent with a dipole model in the vicinity of charge pairs, and selection of peptides for study by experiment and calculation.

Major Findings:

The additional crystal structures available since previous published tabulations of statistical Phi-Psi maps permit substantial improvements in the appearances of these maps. This is manifested in several ways: the maps are substantially smoother and also, there are clearer distinctions between the beta domains of the different residues. Unfortunately there is still not sufficient data to obtain useful maps for many specific pairs of amino acids.

The position dependent statistics of amino acids in secondary regions manifest a number of interesting features not frequently noted before. The most obvious features are the prominence of proline at the amino terminus of helices and the high probability of asparagine at the carbonyl end of beta strands. Numerous other weaker position effects also appear.

Simulations of the interaction energy of ion pairs with a model dipolar solvent indicate that at relatively close distances the energies of interaction of charges of the same and opposite signs are not simply equal in magnitude but opposite in sign as for simple Coulombic interactions. Instead, the opposite signed charges interact more favorably and to longer distances than expected because of the possibility of favorable arrangements of the intervening solvent dipoles.

Investigations of close hydrophobic pairs of amino acids have indicated that they diminish in number monotonically from the center to the exterior of globular proteins. Methods were developed to combine results from large numbers of diverse proteins.

Significance to Biomedical Research and the Program of the Institute:

Development of a reliable method for predicting peptide conformations from their sequences would permit a better understanding of protein and peptide structure and function.

Proposed Course:

We intend to pursue the position dependences of amino acids in secondary regions to develop a method for secondary structure predictions. Further refinements to the dipolar solvent model are intended.

For a few short specific sequence peptides, we will obtain fluorescence intra-molecular distance measurements and 2D NMR distance measurements to combine with calculations of possible conformations. This combination of experimental distance constraints should serve to eliminate sufficient numbers of the large number of conformations and yield average properties of the preferred conformations. In addition we hope to obtain information about the temperature and solvent dependences of such preferences.

Publications:

Church, W. R., Jernigan, R. L., Toole, J., Hewick, R. M., Knopf, J., Knutson, G. J., Nesheim, M. E., Mann, K. G., and Fass, D. N.: Coagulation Factors V and VIII and Ceruloplasmin Constitute a Family of Structurally Related Proteins. Proc. Natl. Acad. Sci. USA 81, 6934-6937, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08323-10 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Assay Quantitation

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigetor.) (Name, title, laboretory, and institute affiliation)*

Charles DeLisi, Ph.D. Chief, Theoretical Immunology Section, LTB, NCI

COOPERATING UNITS *(if any)*

Dr. John Inman, Laboratory of Immunology, NIAID; Dr. Irwin Chaiken, Laboratory of Chemical Biology, NIAID; Dr. Jan Cerny, University of Texas; Dr. Herbert Hethcote, Department of Mathematics, University of Iowa

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

A physical chemical analysis of affinity chromatography has led to the design of new techniques for the quantitative study of macromolecular interactions. In particular new methods have been proposed which should allow rapid, accurate determination of thermodynamic and kinetic parameters. These methods are now being tested experimentally.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives:

To develop a simple, fast, widely available method for obtaining quantitative physical chemical information for complex reaction systems. To develop quantitative methods for obtaining kinetic and thermodynamic information on antigen antibody reactions at a single cell level. To develop methods for increasing assay reliability and precision.

Methods employed:

Mathematical models; mathematical analyses of data.

Major findings:

The chromatography theory is still in its early stages of development, but the equations derived have been applied by Dr. John Inman, NIAID to determine the equilibrium constant for an anti TNP myeloma. He obtains a value within 5% of the accepted value that had previously been determined by dialysis. Work on applications of the plaque assay, especially as a method for analysis of anti-idotypic antibodies, continued but at a slow pace. Most effort assays whose use as replacements for radio immunassays is continuing to increase. Equations were derived that will allow assay optimization including an analysis of error structure of the system.

Significance to Biomedical Research and the Program of the Institute:

The chromatography project is the basic component of projects related to the physical chemistry of cellular recognition and regulation. It will provide the thermodynamic and the kinetic data required to develop a quantitative understanding of cellular regulation. The work on plaques is intimately related to the project on a B cell regulation. It makes possible a method for studying cellular selection. It also provides a potentially new and valuable method for quantitating under appropriate conditions, the anti-idiotypic antibody response. Immunoassays are important, for among other things, the detection and quantitation of low concentration of ligands in the serum. Aside from the ability to detect abnormally low or high values of hormones that are normally present, they are potentially useful in the detection of tumor associated antigens.

Proposed Course:

Discontinued in fiscal year 1984 except for the publication of manuscripts.

Publications:

DeLisi, C., and Hethcote, H.: Quantitative Affinity Chromatography: Theory and Applications. Florida, CRC Press, 1985, 128 pages.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08335-09 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: "Targeting" Liposomes for Selective Interaction with Specific Cells and Tissues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

John N. Weinstein, M.D., Ph.D.	Senior Investigator	LTB, NCI

Other Professional Personnel:

Oscar D. Holton, III, Ph.D.	Expert	LTB, NCI
Jacques Barbet, Ph.D.	Guest Researcher	LTB, NCI

COOPERATING UNITS *(If any)*

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have studied three conceptually different ways of "targeting" liposomes:

(1) Antibody-mediated targeting. We find that antibody-bearing liposomes bind in large numbers to cells which bear the appropriate antigen. However, the bound liposomes are internalized only if endocytosis is possible. Upon endocytosis, liposome-entrapped methotrexate (MTX) can escape from the endocytic apparatus and bind to cytoplasmic dihydrofolate reductase, inhibiting growth of the cell. In the course of these studies, we developed the first heterobifunctional method for coupling antibody to liposomes.

(2) Physical targeting. We have designed "temperature-sensitive" liposomes, which break down and selectively release an entrapped drug in vivo at temperatures achievable by local hyperthermia. These liposomes selectively deliver MTX to mouse tumors in vivo and inhibit their growth.

(3) Compartmental targeting. We have demonstrated the delivery of liposomes and entrapped drug to lymph nodes after subcutaneous and intraperitoneal, injection and have determined cellular sites of localization. These studies have been extended to antibody-bearing liposomes.

Project Description

Objectives:

To investigate the use of liposomes both in cell biology and in clinical therapy. Within this broad context, to (1) explore the use of antigen-antibody interactions to achieve selective association of liposomes with particular cell types; (2) develop synergistic interactions between "temperature-sensitive" liposomal drug carriers and hyperthermic treatment; (3) identify the mechanisms of spontaneous, serum-induced and osmotically-induced release of solutes from liposomes; (4) deliver liposomes and their contents to tumor in lymph nodes -- for nonspecific and antibody-mediated binding.

Non-standard Methods Employed:

(a) Preparation of liposomes, by bath and probe sonication; (b) investigation of liposome-cell interactions using the fluorescence-activated cell sorter; (c) dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory for this purpose; (d) measurements of release of solute from liposomes by "fluorescence self-quenching"; (e) determination of the mobility of vesicles bound to cells, using fluorescence photobleaching (f) microwave heating of tumors; (g) quasi-elastic light scattering measurements of liposome size; (h) covalent coupling of antibodies to liposomes.

Major Findings:

Objective 1: (a) Bivalent antibody selectively binds TNP-bearing liposomes to TNP-bearing lymphocytes, but the binding does not increase delivery of liposome contents to the cell interior (beyond the amount internalized spontaneously). (b) Endogenous surface IgA on cells of the murine myeloma MOPC 315 can bind liposomes bearing the appropriate hapten (DNP) to the cell surface. However, as in the lymphocyte system, binding does not increase delivery to the cytoplasm. (c) Lipid vesicles containing fluorescent molecules are potentially useful as markers for sparse or low-affinity cell surface determinants. They can be made to contain many fluorophore molecules, thus amplifying the signal. They give very low-nonspecific background since the fluorophore is sequestered, and appropriate lipids are not very "sticky". (d) Liposomes are removed from the circulation of a mouse much faster if the mouse carries a myeloma secreting antibody to a hapten on the liposomes. (e) Antibody-mediated binding of methotrexate-containing vesicles to MOPC 315 and TEPC 15 myeloma cells did not lead to entry of drug into the cells and inhibition of their metabolism. (f) IGG opsonized DNP-vesicles are bound in large numbers to F_c receptor-bearing cells (murine $P388D_1$), and are then endocytosed. Liposome-encapsulated MTX can escape the pagolysosomal system to reach a cytoplasmic target (dihydrofolate reductase) and affect the physiology of the cell. (g) IgG, protein A, avidin, and other ligands can be coupled efficiently to liposomes by use of the heterobifunctional cross-linking agent N-hydroxysuccinimidyl 3-(2-pyridyldithio) propinate. This method of coupling results in only minimal aggregation and little leakage of vesicle contents. Liposomes bearing covalently coupled mouse monoclonal antibody against human α_2-microglobulin bind specifically to human cells, but not to mouse cells.

Objective 2: (a) Small uniamellar vesicles of 3:1 dipalmitoylphosphatidylcholine - distearoylphosphatidylcholine release their contents very slowly at 37°C, much faster at 41 - 46°C. Such "temperature-sensitive" liposomes appear useful in achieving high drug concentrations selectively in local areas of hyperthermic treatment, for example, in the treatment of tumors. (b) The temperature-dependence of the release can be enhanced by increasing the rate of temperature change, by using multilamellar vesicles in place of the unilamellar ones, and most markedly, by the presence of serum in the medium. Ratios of greater than 100:1 can be obtained for release (of a fluorescent marker) at 43 and 37°. (c) The effect of serum is largely due to interaction of serum lipoproteins (VLDL, IDL, LDL, and HDL) with the liposomes. (d) Four times as much methotrexate was delivered to subcutaneous Lewis lung tumors heated to 42° as to unheated controls in the same animals at 36°; with L1210 tumor the ratio was 14:1. (e) Growth of the L1210 tumors was delayed by such treatment more than could be accounted for by the separate effects of heating and liposomal drug administered separately. (f) Large unilamellar vesicles are stable below T_c but release their contents within a few seconds upon passage through T_c with serum.

Objective 3: (a) Release of carboxyfluorescein from small unilamellar vesicles takes place by "leakage", not by an all-or-nothing "rupture" of the vesicle. (b) The rate constant for leakage increases in inverse proportion to the hydrogen ion concentration of the medium. (c) Liposomes of dioleoyl lecithin leak their contents and form structures with a characteristic appearance in negative-staining electron microscopy when allowed to interact with HDL or LDL. The interaction is faster and more pronounced with isolated HDL apolipoprotein than with the whole lipoprotein particle. (d) Liposomes bearing the DNP-hapten can be made to release carboxyfluorescein in the presence of complement and IGG anti-TNP. Fluorescence self-quenching provides the most sensitive technique available for continously monitoring such processes.

Objective 4: (a) Liposomes can pass intact (and without release of contents) from the peritoneum to the bloodstream. This finding relates to the possible clinical instillation of liposomes intraperitoneally. (b) Passage from peritoneum to blood takes place largely through the lymphatics. (c) After subcutaneous injection, liposomes pass to regional nodes and then to the bloodstream. (d) The only lymph node cells which take up significant amounts of unmodified liposomes are the macrophages. (e) Some, but not all cellular antigens in the lymph nodes can be targeted by antibody-bearing liposomes.

Significance to Biomedical Research and the Program of the Institute:

The three objectives listed relate to the possibilities of using liposomes in diagnosis and tumor therapy. A major barrier to such efforts has been the difficulty of directing liposomes to particular cells or anatomical sites. The studies of antibody-mediated "targeting" suggest a way to achieve selectivity but also demonstrate an additional problem: how to get the liposome and its contents into the cell after binding. The studies of synergism between liposomes and hyperthermia indicate a new way to achieve selective delivery. Use of the liposome as a hapten-carrier for analysis and sorting of cell populations may be useful in a number of areas of cellular and tumor immunology. The covalent attachment of immunoglobulin and ligands to liposomes will make possible a wide

range of cell biological studies. Liposomes may be able to carry diagnostic and therapeutic agents to tumor in the lymph nodes.

Proposed Course:

This project has been phased-out this fiscal year except for the processing of manuscripts.

Publications:

Chused, T.M., Sharrow, S.O., Weinstein, J.N., Ferguson, W.J., and Sternfeld, M.: XRITC: A new dye for two-color immunofluorescence. J. Histochem and Cytology., in press.

Weinstein, J.N., and Leserman, L.D.: Liposomes as drug carriers in cancer chemotherapy. In I.D. Goldman (Ed.) The International Encyclopedia of Pharmacology and Therapeutics. New York, Pergamon Press, in press.

Klausner, R.D., Blumenthal, R., Innerarity, T., and Weinstein, J.N.: The interaction of apolipoprotein A-I with unilamellar vesicles of L-α-dimalmitoyl phosphatidylcholine. J. Biol. Chem., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08341-07 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Physical Chemical Studies of Lipid - Protein Interactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

John N. Weinstein, M.D., Ph.D. — Senior Investigator — LTB, NCI

Other Professional Personnel:

Robert Blumenthal, Ph.D. — Chief, Membrane Structure & Function Section — LTB, NCI

COOPERATING UNITS *(if any)*: Dr. T. Innerarity and Dr. R. Pitas, University of California at San Francisco; Dr. Richard Klausner, LBM, NIAMDD; Dr. R. Schwartz, LI, NIAID

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have investigated the interaction of lipoproteins with liposomes to form recombinant particles. A number of lipoprotein fractions (VLDL, IDL, LDL, and HDL) all disrupt liposome structure by an essentially irreversible and quasistoichiometric process. In the case of HDL, the major apoprotein, A-I, recombines with dimyristoyl phosphatidyl choline vesicles 40:1 lipid-protein to form discs approximately 100 Å in diameter and 32 Å in thickness, with protein on the rim. These structural results were obtained by a combination of neutron scattering, electron microscopy, column chromatography, and fluorescence techniques.

With dipalmitoyl phosphatidylcholine, A-I also forms what we term "vesicular recombinant" particles in a process which may relate to physiological mechanisms by which proteins are assembled into membranes and lipoproteins. To study this process we have developed a technique called "phase transition release" (PTR) which is also being applied to study incorporation of tubulin into membranes.

Lipoproteins were labelled with the fluorescent lipid 3,3 dioctadecylindocarbocyanine for studies of interaction will cell surface lipoprotein receptors. The lipoproteins are also being labelled with NBD lipids for two-color fluorescence identification of cells in atherosclerotic plaques.

Lipid membrane systems are being used to investigate the interaction between characterized synthetic antigenic peptides and T-cell recognition of antigens.

GPO 914-918

Project Description:

Objectives:

To investigate the interaction between liposomes and lipoprotein and between lipoproteins and cells. More specifically, (1) To define the mechanism by which liposomes are broken down by serum components, principally the lipoproteins; (2) to determine the relative efficacies of different purified apolipoproteins in breaking up liposomes; (3) to develop fluorescently labelled lioporoteins and liposome-apoprotein recombinants for use in studying lipoprotein-cell interactions; (4) to use the methods developed for objectives (1) - (3) to assess the physical chemistry of protein-lipid interaction in lipoproteins; (5) to extend to other bilayer-protein interactions the concepts thus developed; (6) in particular, to investigate the assembly of tubulin and actin into membranes; (7) to study physical chemical aspects of T-cell antigen receptor function.

Non-standard Methods Employed:

(a) Preparation of liposomes, by probe and bath sonication: (b) Dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory, i.e., "phase transition release" (PTR); (c) Determination of the mobility of lipoproteins and liposomes bound to the cells, using fluorescence-photobleaching recovery; (d) Neutron scattering studies of lipoprotein size and shape; (e) Aqueous collisional fluorescence quenching studies of lipid-protein interactions; (f) Derivatization of proteins with trinitrobenzene sulfonate; (g) Labelling of lipoproteins with 3,3'-dioctadecylindocarbocyanine (diI) dye.

Major Findings:

(1) Liposomes are broken down and their contents released by an interaction with serum, most rapidly at the lipid phase transition. (2) The serum effect is mostly due to low density (LDL) and high density (HDL) lipoproteins, but very low density and intermediate density lipoproteins also play a part, as also does some other, unidentified component of the serum. (3) Heparinized plasma, EDTA plasma, and serum all have the same effect. (4) At least in the case of apo-HDL the mechanism appears to be a quasi-stoichiometric, all-or-nothing breakdown of the vesicle into a small disc of lipid rimmed with protein. The process is largely complete within a second or two at the phase transition of the liposome lipid and is essentially irreversible on that time scale. (5) By neutron diffraction and electron microscopic studies the discs appear to be about 100 Å in diameter and 32 Å in thickness, with a protein rim. They probably consist of a single bilayer. (6) Cholesterol at 40 mole percent in the liposomes severely restricts the interaction with serum components, as does the formation of liposomes from lipids which are below their phase transitions at the temperature of study. (7) At molar ratios of about 2,000:1 dipalmitoyl phosphatidylcholine:Apo A-I, a stable vesicular recombinant particle (VR) is formed below the lipid phase transition temperature (Tc). As the temperature is raised through Tc (in PTR), a new type of recombinant (VR-Tc) is formed. By physical measurements of several types, the A-I's conformation and disposition in the lipid change at Tc. The protein probably becomes trans-membrane. These findings may relate to physiology

processes for formation of HDL and to the assembly of intrinsic proteins into membranes. (8) Purified tubulin interacts with vesicles at Tc to form vesicular recombinants. The interaction is accompanied by structural changes in lipid and protein. (9) HDL, LDL, and apoE-HDL can all be labelled efficiently and irreversibly with the fluorescent lipid analogue, diII. The lipoproteins are unchanged in physical properties and specific binding to cell surface receptors. The labelled lipoproteins were acetoacetylated for studies of phagocytic and lipoprotein-specific uptake in arterial walls. (10) Studies of the interaction with bilayers of a hepatic membrane receptor for asialoglycoprotein have been described in another report.

Significance to Biomedical Research and the Program of the Institute:

(1) A major barrier to the effective use of liposomes as carriers in cancer chemotherapy and diagnosis has been a lack of understanding of their interactions with serum. Our studies define the interaction and indicate what type of liposomes must be used to avoid it. (2) Our studies of HDL-liposome recombinants contribute to (1) and may also be useful in delineating mechanisms of atherosclerosis. Our fluorescently labelled lipoproteins are currently being used by collaborators to study atherogenesis. (3) Breakdown of liposomes in serum is essential to the combination of liposomes with hyperthermia to achieve selective release of drugs in the area of a tumor. (4) Antigen presentation to T-lymphcytes is an important function within the immune system.

Proposed Course:

This project was terminated during FY 1984, except for the work on antigenic pepdides and the processing and publication of manuscripts.

Publications:

Klausner, R.D., Blumenthal, R., Innerarity, T., and Weinstein, J.N.: The interaction of apolipoprotein A-I with unilamellar vesicles of L-α-dipalmitoyl phosphatidylcholine. J. Biol. Chem., in press.

Weinstein, J.N., Blumenthal, R., and Klausner, R.D.: Carboxyfluorescein leakage assay for lipoprotein-liposome interaction. Methods in Enzymology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08357-04 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell Interactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Charles DeLisi, Ph.D. Chief, Theoretical Immunology Section LTB, NCI

Other Professional Personnel:

David Covell, Ph.D. Senior Staff Fellow LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Richard Asofsky, Laboratory of Microbial Immunity, NIAID
Dr. Jerome Eisenfeld, Dept. of Mathematics, Univ. of Texas at Arlington

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Helper and suppressor cells form feedback loops which regulate the immune response, and account for the central phenomena of immunology such as tolerance and maturation. We have developed important theoretical criteria involving control loop stability which tells us whether the loops thus for identified loops can in fact explain phenomena of interest.

GPO 914-918

Project Description:

Objectives:

To develop criteria which allows a determination of the qualitative stability of models.

Major Findings:

We have found that the most popular model of immune response regulation; viz the model of Herzenberg et al. is qualitatively unstable; i.e. the model is unstable irrespective of parameter values or the forms of the functions controlling the relation between different cells of the model. It is therefore biologically untenable. An alternative model has been proposed which is stable. It is currently being analyzed to determine if it accounts for phenomena of interest.

Significance to Biomedical Research and the Program of the Institute:

This project places in perspective the extent to which current experimental findings contribute to our understanding of immune response regulation. In that way it helps filter the findings with major implications from other less important findings.

Proposed Course:

Our current model will be analyzed and developed further if necessary to account for tolerance, memory, and maturation. Its predictions will be simulated and tested experimentally.

Publications:

Eisenfeld, J., and DeLisi, C.: On conditions for qualitative instability of regulatory circuits with applications to immunological control loops. In Eisenfeld, J., and DeLisi, C. (Eds.): Mathematics and Computers in Biomedical Applications. Amsterdam, Elsevier, 1985, pp. 39-53.

Eisenfeld, J., and Delisi, C. (Eds): Mathematics and Computers in Biomedical Applications. Amsterdam, Elsevier, 1985, 389 pgs.

Covell, D.C., Berman, M. and DeLisi, C.: Calculation and use of mean residence time in kinetic analysis. In Eisenfeld, J., and DeLisi, C. (Eds.): Mathematicas and Computers in Biomedical Applications. Amsterdam, Elsevier, 1985, pp. 269-273.

Covell, D.C., Berman, M. and DeLisi, C.: Mean residence time -- Theoretical development, experimental determination and practical use in tracer analysis. Math. Biosci. 72: 213-244, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08359-04 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Monoclonal Antibodies in the Lymphatics for Diagnosis and Therapy of Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

John N. Weinstein, M.D., Ph.D.	Senior Investigator	LTB, NCI
Other Professional Personnel:		
David G. Covell, Ph.D	Senior Staff Fellow	LTB, NCI
Oscar D. Holton, III, Ph.D.	Expert	LTB, NCI
Jacques Barbet, Ph.D.	Guest Worker	LTB, NCI
Mary J. Talley	Biologist	LTB, NCI
Christopher D.V. Black, Ph.D.	Visiting Fellow	LTB, NCI
Renee Eger, B.S	Guest Researcher	LTB, NCI

COOPERATING UNITS *(if any)* Dr. A. Keenan, Dr. S.M. Larson, LHM, CC; Dr. R. Parker, Dr. S. Sieber, DCCP; Dr. R.K. Oldham, Dr. K.M. Hwang, Dr. M.E. Key, FCRF; Dr. L. Liotta, Dr. G. Bryant, LP, DCBD; Dr. J. Schlom, Dr. D. Colcher, LTIB, DCBD; Dr. M. Lotze, Dr. R. Rosenberg, SB, DCT; Dr. J. Mulshine, NCI-NMOB, DCT.

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have defined a new approach to the use of monoclonal antibodies for diagnosis and therapy of tumor in lymph nodes: delivery to the nodes via lymphatic vessels after subcutaneous injection. To establish a firm pharmacokinetic basis for this approach, we first studied antibodies to normal cell types in the mouse lymph node. In vitro binding characteristics were combined with in vivo pharmacological parameters to develop a quantitative understanding of the delivery process using the SAAM computer modeling system. Armed with that background information, we then demonstrated and analyzed specific uptake in lymph node metastases of a guinea pig tumor. Imaging studies were followed up with attempts at therapy. For diagnosis of early metastatic tumor in the nodes, the lymphatic route can be expected to provide higher sensitivity, lower background, lower systemic toxicity, and faster localization than the intravenous route. It will also minimize the problem of cross-reactivity with antigen present on normal tissues.

The experimental design of the guinea pig studies is currently being applied to detection of lymph node metastases in clinical stage II malignant melanoma, breast cancer, and cutaneous T-cell lymphoma (CTCL). A similar protocol has been approved for non-small cell lung cancer. Our studies of CTCL have produced the most efficient imaging of tumor cells yet achieved in humans by any techniques.

In vitro and animal studies are being continued both to optimize the clinical procedures and to explore basic functions of the immune system (see project #Z01CB08368-2 Selective Cytotoxicity in the Lymphatics). Our longer term aim is to understand the pharmacology of monoclonal antibodies and other ligands in order to develop criteria for rational molecular design of biological antitumor agents.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives:

(i) Establish a quantitative basis (experimental and theoretical) for lymphatic delivery of monoclonal antibody to lymph nodes; (ii) Use that information for optimization of diagnostic imaging of animal tumors; (iii) Treat the tumors with radio-labeled and/or toxin-labeled antibodies (using experimental parameters obtained from the diagnostic studies); (iv) Extend the principles of lymphatic immunodiagnosis and immunotherapy to the clinical setting; (v) Extend our theoretical and experimental models to the intravenous administration of monoclonals; (vi) Apply the lymphatic approach to detection of lymph node metastases of malignant melanoma, breast carcinoma, lymphoma, non-small cell lung carcinoma, and other malignancies; (vii) Extend the lymphatic delivery of monoclonal antibodies to therapeutic modalities; (viii) Investigate the lymphatic pharmacology of immunization and toleragenesis.

Non-Standard Methods Employed:

(a) Two-color fluorescence microscopy and fluorescence cell-sorting on cell populations labeled in vivo with monoclonal antibodies; (b) Image-correction techniques for quantitative analysis of gamma camera studies; (c) Use of SAAM modeling system to analyze the pharmacokinetics of antibody distribution and binding in vivo; (d) Filtration and centrifugation methods for determination of equilibrium and kinetic binding parameters for monoclonal antibodies; (e) Labeling of antibodies for imaging by nuclear magnetic resonance; (f) Autoradiography of diffusible antibodies; (g) Immunohistochemistry.

Major Findings:

Antibodies directed against normal cells:

(1) Antibodies against normal cell types (in the mouse and guinea pig) can be delivered with high efficiency from subcutaneous injection sites to regional lymph nodes. The normal nodes can be imaged with the gamma camera if the antibodies are radiolabeled with I-125 or I-131. (2) Injections can be made so as to label all cells of the target population in the node. (3) Pharmacokinetic experiments and modeling indicate that correct choice of dose, volume, osmolarity, and site of injection are all important if efficiency of the imaging technique is to be maximized. These findings suggest clinical use of antibodies against normal lymphoid cells as an alternative to colloid lymphoscintigraphy for diagnostic imaging of lymph nodes.

Antibodies directed against tumor cells:

(1) Antitumor antibodies can be delivered with high efficiency from subcutaneous injection sites to regional lymph nodes (in the guinea pig). (2) Early metastatic tumor in the lymph nodes is selectively labeled with the antibody, and gamma camera images of the tumor deposits can be obtained. (3) The smallest

amounts of tumor imaged in this way are about 3 mg (much smaller than has been detected by i.v. monoclonal antibodies or other non-invasive methods of detection). (3) The double-label experimental design developed for animal studies is appropriate for use in clinical trials of lymphatic delivery of monoclonal antibodies. (4) Antibodies against normal lymphoid cells can be used in tandem with antibodies against tumor. The former indicates gross characteristics of the node and/or its immunological status; the latter detects metastatic tumor with high sensitivity. (5) Subcutaneous injections of T101 antibody.

Significance to Biomedical Research and the Program of the Institute:

In the staging of metastatic tumors, it is almost always necessary to assess the status of lymph nodes. Current non-invasive techniques (standard x-ray, computerized axial tomography, lymphangiography) are usually limited in sensitivity to the detection of masses larger than 1 cubic cm. In guinea pigs we detected metastases 300 times smaller. If some portion of that sensitivity could be obtained clinically, the need for surgical assessment of nodes (e.g., in breast cancer) might be reduced. More speculatively, antibodies armed with toxins or drugs might be used to treat lymph node metastases. By a combination of in vitro cell experiments, animal studies, theoretical modeling, and clinical studies, we are exploring these possibilities.

The lymphatic route for administration of monoclonal antibodies may be useful as well for understanding and manipulating the regional immune response. These issues are significant from the point of view of sensitization and tolerance to tumors.

Proposed Course:

(1) Continue to develop systems for in vivo delivery of monoclonal antibodies to T- and B-cell sub-populations in the mouse lymph node, both for refinement of the approach to diagnosis and therapy of lymph node tumor and as a means of modulating immune function; (2) Continue to use the line 10 tumor of guinea pigs to improve strategies for clinical detection and treatment of lymph node metastases; (3) Continue study of clinical stage II melanoma patients with Dr. Steven Larson; (4) Extend the clinical studies to lymphoma (with Dr. P. Bunn and co-workers), breast carcinoma (with Dr. J. Schlom and co-workers), and non-small cell lung carcinoma (with Dr. J. Mulshine and co-workers); (5) Extend the studies to therapy via immunotoxins, complement, and radioconjugates (see project #Z01CB08368-01); (6) Continue theoretical and experimental studies of the molecular cell biology of antibody interaction with cell surface antigens and endocytic machinery.

Publications:

Weinstein, J.N., Parker, R.J., Holton, O.D. III, Keenan, A.M., Covell, D.G., Black, C.D.V. and Seiber, S.M.: Lymphatic delivery of monoclonal antibodies: Potential for detection and treatment of lymph node metastases. Cancer Investigation 3: 85-95, 1985.

Chused, T.M., Sharrow, S.O., Weinstein, J.N., Ferguson, W.J., and Sternfeld, M.: XRITC: A new dye for two-color immunofluorescence. J. Histochem. and Cytology, in press.

Weinstein, J.N., Keenan, A.M., Holton, O.D. III, Covell, D.G., Sieber, S.M., Black, C.D.V., Barbet, J., Talley, M.J., and Parker, R.J.: Use of Monoclonal antibodies to detect metastases of solid tumors in lymph nodes. Monoclonal Antibodies and Breast Cancer, in press.

Weinstein, J.N., Black, C.D.V., Keenan, A.M., Holton, O.D. III, Larson, S.M., Sieber, S.M., Covell, D.G., Carrasquillo, J., Barbet, J., and Parker, R.J.: Use of monoclonal antibodies for detection of lymph node metastases. In Monoclonal Antibodies in Medicine (ICN-UCLA Symposium), in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08363-03 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Protein Modelling

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

H. Robert Guy, Ph.D.	Expert	LTB, NCI
Other Professional Personnel:		
Robert Jernigan, Ph.D.	Theoretical Physical Chemist	LTB, NCI
Peddaiaghari Seetharamulu, Ph.D.	Visiting Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	2.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Molecular models of the action potential channel, interleukin 2, δ hemolysin, and α toxin were developed. This was done by analyzing their sequences using methods previously developed to predict which portions of α helices and β strands should be exposed to water, other protein segments, or in membranes to hydrocarbon lipid chains. The method was also used to analyze possible homologies of distantly related proteins. An interesting finding is that one face of an amphipathic α helix appears to be conserved in human granulocyte-macrophage colony stimulating factor, somatotropin, lactogen, and the E peptide of insulin-like growth factor II. The globin superfamily of sequences, as a prototype of helical proteins, was analyzed to learn which aspects of their structures were most conserved, as well as to determine, on such basis, the relationships between distantly related globin sequences. The most conserved parameter is the partition energy for moving α helical segments from water to their optimal positions at the water-protein interface. The study indicated that leghemoglobins are almost equally related to vertebrate and invertebrate globins, therefore suggesting an evolutionary branch from these globins corresponding to the origin of animals from plants.

Project Description

Objectives:

The major objectives are to develop methods to predict protein structures and facilitate modelling their conformations. These methods can be applied to identify homologous proteins from their sequences. It is well known that residues of protein surfaces that are exposed tend to be more polar than those buried inside proteins and that residues exposed to the apolar lipid phase of membranes tend to be less polar than those buried inside proteins. We have been developing methods to quantify energies responsible for these effects and schemes to predict form sequences which residues are exposed to water, buried inside proteins, exposed to membrane lipids or located near water-protein or protein-lipid interfaces. These calculations have been combined with other factors to predict secondary structure, to locate possible transmembrane segments, to evaluate the amphiphilicity of α helices and β sheets, to identify homologous protein segments from the physical properties of their amino acid residues, and to develop molecular models of proteins of unknown structure, principally membrane proteins.

Methods Employed:

Equations have been developed to calculate the energy required to transfer a residue from water to any point in a protein based on the distance of that point form the water-protein interface. These equations are based on statistical analyses of residue distributions in proteins of known structure and on partitioning of amino acids between water and organic solvents. Similar relationships have been developed for protein-lipid interfaces based on partitioning of amino acid side chain analogs between water and organic solvents. These relationships have been used to predict the optimal orientation of protein segments of fixed conformation at water-protein, protein-lipid, and water-lipid interfaces. These energies required to transfer the segments from water to their optimal position can be used with other factors to predict secondary structure and the environment to which portions of a structure are exposed. The method is tested by analyzing proteins of known structure. Predictions made using this method are used as an initial step in developing molecular models of membrane proteins. The simplest sequence we have been modeling is that of δ hemolysin. We have also been analyzing this sequence with the empirical conformation energy program for peptides (ECEPP) to include other energy factors in our model building.

Major Findings:

Aspects of the nicotinic acetylcholine receptor (AChR) model that was developed previously have been tested in several laboratories using antibodies to specific segments and single site mutagenesis experiments. Most important aspects of the model have been confirmed by these experiments. The theory used for the AChR model has been used to develop a model of the other most studied membrane channel of the nervous system, the action potential sodium channel. The sodium channel model is similar to the acetylcholine receptor channel model in that most

transmembrane segments are α helices. The model was developed to explain the molecular mechanism of voltage-dependent channel activation.

The mechanism by which interleukin 2 (IL-2) stimulates division of T cells probably involves association of kinase C with the plasma membrane but is not well understood. Analysis of the IL-2 sequence indicated that its COOH terminal segment may form an amphipathic α helix that could interact with membrane lipids and possibly aggregrate to form a membrane channel. This hypothesis is being tested in Robert Blumenthal's laboratory. Initial results indicate that IL2 increases the permeability of lipid vesicles in a voltage and pH dependent manner predicted by the model.

A number of proteins may stimulate cell division by a mechanism similar that of IL2. Sequences of colony stimulating factors, interleukin 3, somatotropin, latogen, prolactin, and proliferin were analyzed with the program that predicts solvent exposures of α helices and β strands. All of these factors and hormones contain segments that may form amphipathic α helices that could interact with membrane lipids. One face of a potential α helix appears to be conserved in granulocyte-macrophage colony stimulating factor (GM-CSF), somatotropin, lactogen, and the E peptide that is cleaved form the precursor to insulin-like growth factor II (IGF II).

α toxin and δ lysin are secreted by staphyloccus to lyse cell membranes, probably by forming membrane channels. Analysis of δ lysin with ECEPP predicts that the central portion of this 26 residue peptide should form an α helix. The fraction of the sequence predicted to be helical is almost identical to the fraction calculated from ORD experiments. Analysis of the α toxin sequence indicated that there should be no transmembrane α helices and that the channel wall is probably formed by amphipathic β strands. This conclusion is consistent with structural studies.

Significance to Biomedical Research and the Program of the Institute:

A major goal in conformational studies is the prediction of protein conformations from their sequences. These types of studies have been stimulated by the recent availability of methods to easily determine and modify sequences.

The sodium channel is one of the most basic and ubiquitous membrane channel of the nervous system. Understanding its structure and mechanism of opening and closing may lead to important discoveries about drugs and toxins that affect nervous systems.

Several protein toxins form membrane channels. We have analyzed sequences of two staphylococcus toxins and have develped molecular models of their structures. These models may help us understand molecular mechanisms of staphyloccal infections specifically and mechanisms of toxin interactions with membranes in general.

It is possible that there may a connection between cancer and malfunctions of normal mechanisms that stimulate cell division. Also, agents that stimulate division of immune system cells could be important in treating cancers and other

diseases. It is thus important to understand the basic mechanisms of these processes. Interleukins, colony stimulating factors and protein hormones have generally been regarded as soluble proteins that bind to membrane receptors but do not interact directly with membrane lipids. Our theoretical prediction that IL-2 may bind to lipids has been experimentally confirmed. Structural similarities among colony stimulating factors, insulin-like growth factor II (IGF II) precursor, hormonal proteins, and interleukins are interesting for several reasons. The family of homologous hormones that include somatotropin, prolactin, and lactogen affect the immune system in a way that is not well understood. The apparent homologous helix faces on GM-CSF and somatotropin and/or lactogen may bind to the same receptor. There is a variant of human somatotropin with a deletion in the region of the apparent homology; its effect appears similar to that of somatotropin except for its insulin like activity. Secretion of IGF II is stimulated by lactogen. The E peptide that is cleaved from the IGF II precursor may act as a feedback regulator by binding to the lactogen receptor. Proliferin is closely homologous to prolactin and slightly less homologous to somatotropin. Synthesis of proliferin by 3T3 fibroblastic cells is stimulated by serum or platelet-derived growth factor. These homologies suggest that mechanisms used by colony stimulating factors to induce cell divisions may be related to those used by some oncogene products and hormonal proteins.

Proposed Course:

The methods of using the partition energy calculations to analyze homologies of distantly related proteins need to be introduced into a more rigorous computer algorithm and made more quantitative. We have begun to use ECEPP to make our models of δ lysin more quantitative and precise. We hope to extend these methods to the larger proteins we have been studying upon the advent of larger computers. It is important to collaborate with experimentalists to test these models. Studies of membrane interactions of IL-2 in Dr. Blumenthal's laboratory will continue and may be extended to other agents that we study. We have tentatively located a group to test the prediction that GM-CSF and somatotropin and/or lactogen bind to the same receptor. Predictions related to the sodium channel model are being tested. Following the success or failures of these predictions, we will modify methods and proceed to further models that should be more realistic.

Publications:

Guy, H. R.: Amino Acid Side-chain Partition Energies and Distribution of Residues in Soluble Proteins. Biophys. J. 47: 61-70, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08364-03 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Quantitative Methods for Analyzing Receptor Mediated Binding and Endocytosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Charles DeLisi, Ph.D. Chief, Theoretical Immunology Section LTB, NCI

Other Professional Personnel:

Marianne Gex-Fabry, M.Sci., Visiting Associate LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A compartmental model has been developed for the analysis of receptor mediated endocytosis. It considers ligand binding to receptors, diffusion at the cell surface, interaction of ligand-receptor complexes with coated pits, internalization of coated pit contents, lysosomal degradation and recycling to the surface.

The model makes a number of predictions related to the interpretation of binding data. It has been tested against, and applied to the analysis of, a large body of data on binding and endocytosis of peptide hormones and modulation of the effects of growth factors by tumor promotors.

PHS 6040 (Rev. 1/84) GPO 814-916

Project Description

Objectives:

To develop a sound physical chemical framework for interpreting data on receptor mediated endocytosis and related processes.

Methods Employed:

Mathematical modelling

Major findings

1. The model permits quantitative characterization of the steps in binding, endocytosis, degradation and recycling of ligands. It accurately simulates and quantitatively fits a wide range of data and can thus be used to aid in the design of new experiments. It identifies the variables that account for the behavioral differences between different receptor-ligand systems (e.g. receptor recycling vs. segregation), as well as those within a particular system.

2. Our analysis indicates that the wide variation in linear Scatchard plot shapes that were previously attributed to differences in ligand receptor affinties in different cells is likely due to events distal to binding. Candidates for these events have been identified.

3. The model shows that variations in the protein composition of coated pits can result in variation of the shape of Scatchard plots. Thus the observed variations in the Scatchard plot slopes for EFG systems, (sometimes linear; sometimes non-linear) need not imply several classes of EGF receptors for some cells and only one class for others.

4. The model explains the loss of the "high affinity" component of the EGF Scatchard plot when cells are pre-incubated with phorbol esters, in terms of competition for a common effector (coated pit) protein. This not only provides a much simpler more quantitative interpretation of literature data than previous hypotheses, but predicts an as yet to be discovered local sequence homology between the EGF receptor and phorbol ester receptor.

Significance to Biomedical Research and the Program of Institute:

The development of quantitatively sound methods for accurate characterization of receptor-ligand interactions and receptor endocytosis is important for the rational design of protocols for detection and treatment of tumors by surface antigen specific ligands.

Proposed Course:

Discontinued in fiscal year 1984 except for the publication of manuscripts.

Publications:

Gex-Fabry, M., and DeLisi, C: Kinetic and Steady state Analysis of Receptor mediated endocytosis. II. Tumor promotor growth factor modulation. Math Biosci., 72: 245-261, 1984

Gex-Fabry, M., and DeLisi, C.: Receptor mediated endocytosis: A mathematical model and its implications for experimental analysis. Am. J. Physiol., 16: R678-R779, 1985.

Gex-Fabry, M., and DeLisi, C.: Modulation of binding and endocytosis of growth factors by phorbol esters II. Amer. J. Physiol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08365-03 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Prediction of Protein Structure, Function and Cellular Location

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Charles DeLisi, Ph.D. Chief, Theoretical Immunology Section LTB, NCI

Other Professional Personnel:

Minoru Kanehisa, Ph.D. Visiting Scientist LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Jay Berzofsky, Metabolism Branch, DCBD, NCI; Dr. Ronald Schwartz, Laboratory of Immunology, NIAID; Dr. Petr Klein, Div. of Biological Sciences, National Research Council, Ottawa, Canada

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have developed a method which uses a series of discriminant analyses to allocate a protein sequence of unknown function to one of 28 functional groups (toxins, immunoglobulin variable regions, cytochromes c, etc.). Allocation is based on characteristics of both global and local physical properties (hydrophobicity, charge, etc.) of the amino acid sequence, and also on the appearance in the sequence of some characteristic patterns, such as repeated consecutive appearance of certain residues, or short signature peptides. A similar method can be used to predict the cellular location of a protein segment.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To develop statistical methods that will allow prediction of the structure, function and cellular location of an amino acid sequence from local and global properties of the sequence.

Methods Employed:

Computational, statistical, physical-chemical analysis.

Major Findings:

The protein database can be segmented into families of sequences on the basis of generic biological functions. We have developed a method that utilizes consensus properties of sequences to determine the functional family to which a newly identified sequence belongs. At the present time, with the database divided into 28 functional groups, the method allocates sequences with reliabilities ranging from 87-100%.

The same statistical technique can be used to predict the location of a sequence in a cell. For example, we can currently distinguish extrinsic from intrinsic membrane proteins with complete reliability. In developing these methods we have identified physical variables which are important correlates of structure and which we expect will be of great value as we develop structural prediction algorithms. We can also distinguish segments internal to a membrane from segments internal to a globular protein. The method is currently being extended to structural classification. Although this work has only just begun, we have found that on the basis of sequence alone, we can predict with perfect reliability whether a protein is α rich β rich.

Significance to Biomedical Research and the Program of the Institute:

Over three million nucleotides have now been sequenced. In the vast majority of cases, the deduced protein sequences have not been biochemically identified; i.e. neither their function, their location nor their structure is known. Some of these proteins are, expected to be of great importance in the control of normal cell behavior and probably also play a role in transformation. The methods we are developing will allow predictions of the location and function of such proteins.

Proposed Course:

Increased emphasis will be placed on refining positional predictors. We would like to be able to distinguish for example, between various categories of integral proteins (channels, pumps, receptors, etc). We also plan to include other cellular locations and thereby gain insight into, how molecules sort within a cell. As the database grows we will continue to increase the number of functional catagories, thereby increasing the precision of these methods.

As we gain more insight into the general principles governing structure-function relations we will continue to introduce additional variables and thus increase the reliability of the method. Greater emphasis will also be placed on structural prediction.

Publications:

DeLisi, C. and Kanehisa, M.: Assessing the signal and local homologies. Math. Biosci. 69: 77-85, 1984.

Klein, P., Kanehisa, M., and Delisi. C.: Prediction of protein function from sequence properties: Statistical analysis of a database. Biochim. Biophys., Acta, 787: 221-226, 1984.

Kanehisa, M. and DeLisi, C.: Sequence homologies and oncogene proteins: A critical review. In Robert Rein (Ed.): Molecular Basis of Cancer, (Part A), New York, H.H Reinhardt, 1985, pp. 443-452.

DeLisi, C., Klein, P. and Kanehisa, M.: Some comments on protein taxonomy: Procedures for functional and structural classification. In Robert Rein (Ed.): Molecular Basis of Cancer, (Part A), New York, H.H Reinhardt, 1985, pp. 431-441.

Kanehisa, M. and DeLisi, C.: Prediction of protein and nucleic structure problems and prospects. Acta Applicanda Mathematica, in press.

Klein, P., Kanehisa, M., and DeLisi, C.: The detection and classification of membrane spanning proteins. Biochim. Biophys. Acta, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08366-02 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: The Percolation of Monoclonal Antibodies into Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

John N. Weinstein, M.D., Ph.D.	Senior Investigator	LTB, NCI
Other Professional Personnel:		
David G. Covell, Ph.D	Senior Staff Fellow	LTB, NCI
Jacques Barbet, Ph.D.	Guest Researcher	LTB, NCI
O. Dile Holton, III, Ph.D.	Expert	LTB, NCI

COOPERATING UNITS *(if any)*: Dr. L. Liotta, LP, DCBD; Dr. S.M. Larson, NM, CC; Dr. B. Bunow, LAS, DCRT

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Before a monoclonal antibody (or other biological ligand) can label or kill a tumor cell, it must first reach that cell. For portions of a tumor far from the nearest blood vessel or other source of antibody, access may be limited by the rate at which the molecule can "percolate" through the extracellular space. We are investigating the spatial and temporal profiles of immunoglobulin (Ig) distribution generated by diffusion and convection through tumors, taking into account the possibilities of (a) saturable specific binding to cells, (b) nonsaturable, nonspecific binding, and (c) metabolic degradation.

We first developed theoretical models of the percolation process, using a VAX 11/780 computer and a program package for numerical solution of partial differential equations. Significant predictions thus far include the following: (1) The diffusion coefficient and/or hydraulic conductivity may limit flux of antitumor Ig through tumors. (2) The flux of non-binding control Ig is much less likely to be limited by diffusion or convection. Nonspecific Ig's penetrate more deeply and more quickly into the tumor. (3) Even with saturable binding (but not metabolism), the "C times T" exposure of tumor cells to antibody will be the same throughout the mass. (4) Metabolism will decrease the relative "C times T" exposure of cells farther from the source. This may be a major barrier to effective treatment of solid tumors with ligand molecules (or, for that matter, with standard chemotherapeutic agents).

We are testing predictions of the model using human melanoma cells injected i.v. in nude mice to form metastatic nodules. The distribution of antibody will be determined by fluorescence techniques and autoradiography. Concepts arising from this study are being applied to the design of clinical studies with monoclonal antibodies.

In addition to the investigations of immunoglobulin and other ligands as administered agents, we are considering the the physiology of endogenous molecular species including the antibodies, lymphokines, and other growth factors.

Project Description

Objectives:

(1) To investigate factors which limit the access of monoclonal antibodies and other ligands to target cells within tumors; (2) To investigate analogously limited access of physiological ligands (e.g., growth factors) to cells within tumors.

Non-standard Methods Employed:

(1) Fluorescence-labeling and radio-labeling of monoclonal antibodies; (2) Double-label autoradiography of diffusible substances; (3) Computer simulation of convection-diffusion equations with non-linear binding, using the PDE-COL program; (4) Use of the SAAM compartmental modeling system to determine boundary conditions for distributed models; (5) In vitro growth of small cell lung carcinoma as spheroids; (6) Intravenous metastasis model in nude mice.

Major Findings:

(1) For physiologically reasonable ranges of parameters, the diffusion coefficient and/or hydraulic conductivity may limit flux of antitumor antibodies through tumors; (2) The flux of non-binding control antibody is much less likely to be limited by the rate of diffusion or convection through the tumor. Such antibodies are predicted to penetrate more deeply and more quickly into the tumor; (3) In the presence of saturable binding but not metabolism, the "C times T" exposure of tumor cells to antibody will be the same throughout the mass. However, the period of exposure will be later for cells farther from the source of antibody; (4) Metabolism will decrease the relative CxT exposure of cells farther from the source. This may be a major barrier to effective treatment of solid tumors with ligand molecules (or, for that matter, standard chemotherapeutic agents).

Significance to Biomedical Research and the Program of the Institute:

(1) For rational planning of diagnostic imaging procedures with monoclonal antibodies and other ligands, it is necessary to investigate the spatial and temporal profiles created by diffusion, convection, and binding; (2) For planning of therapy with ligands, it is, likewise, important to understand the way in which the molecule distributes throughout a tumor; (3) In order to understand the function of endogenous biological ligands with respect to established tumors, it is likewise necessary to know how the ligand molecules distribute.

Proposed Course:

(1) Continue theoretical studies of the convection and diffusion of antibodies into tumors, with saturable binding and metabolism included; (2) Incorporate more complex binding kinetics (for monovalent vs. divalent binding) to compare the characteristics of IgM, IgG, $F(ab')_2$, and Fab; (3) Extend the studies to include the simultaneous presence of competing ligands; (4) Do correlative experiments in vitro using culture-grown spheroids of small cell lung carcinoma in conjunction

with monoclonal antitumor antibodies (with J. Mulshine, A. Gazdar, Bethesda Naval/NCI Oncology program); (5) Do correlative experiments in vivo using i.v. injection of human melanoma cells to develop lung and liver metastases in nude mice. The mice will be injected with I-131-labeled antitumor antibody and I-125-labeled control antibody for determination of the distribution by double-label autoradiography. Analogous experiments will be done with fluorescently labeled antibody.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08367-02 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Selective Cytotoxicity in the Lymphatics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Christopher D.V. Black, Ph.D.,	Visiting Fellow	LTB, NCI

Other Professional Personnel:

Jacques Barbet, Ph.D.,	Guest Researcher	LTB, NCI
O. Dile Holton, III, Ph.D.,	Expert	LTB, NCI
John N. Weinstein, M.D., Ph.D.,	Senior Investigator	LTB, NCI

COOPERATING UNITS *(if any)*: Drs. R.J. Parker and S.M. Sieber., OD, DCE, NCI; Dr. O.A. Gansow and R.W. Atcher., COP, DCT, NCI; Drs. R.A. Kroczek and E.M. Shevach., LI, NIAID

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Following subcutaneous injection, radiolabeled monoclonal antibodies bind efficiently to normal and tumor target cells in the lymph nodes (Project # Z01 CB 08359-02 LTB). This finding prompted us to attempt specific therapy using monoclonal antibodies covalently coupled to plant toxins. The first development along these lines has been to synthesize monoclonal antibody-ricin A-chain conjugates using four antibodies of different specificities. We then demonstrated the capacity of these conjugates to bind to their target cells and to inhibit protein synthesis at the ribosomal level in an acellular system. Unfortunately, using the guinea pig hepatocarcinoma cell line (L 10), which expresses large quantities of target antigen, we found only weak cytotoxicity with the monoclonal antibody D3 coupled to ricin A-chain.

However, the same toxin coupled to an anti-mouse MHC antibody has proved to be highly toxic for lymphoid cells and similar results have been found with ricin A-chain conjugated to monoclonal antibodies against subsets of mouse lymphocytes in vitro. We have also been able to augment the effects of these toxins by the action of certain drugs which are known to affect cell biological processes.

Another approach to specific therapy within the lymphatic system is the subcutaneous injection of a monoclonal antibody followed by a similar injection of complement. Such a system attempts to reproduce physiological antibody/ complement dependent cytotoxicity. The determination of optimal doses and injection regimes will be facilitated by our current studies on monoclonal antibody pharmacokinetics and by in vitro cytotoxicity assays.

A further modality for specific cell killing in vivo is to use radioactive compounds attached to antibodies which, on binding to cell surfaces, can damage the cells by radiation. We have been able to demonstrate that lymph-node cell ablation occurs in mice injected subcutaneously with an anti-murine B cell antibody labelled with the alpha particle emitter bismuth 212. Studies are in progress to optimize this system and to reduce the non-specific toxicity.

Project Description

Objectives:

To investigate the ability of monoclonal antibodies to kill specific cells within the lymphatic system. More specifically:

1) To couple monoclonal antibodies chemically to plant toxins, principally ricin or its catalytic sub-unit, and to determine whether this chemical modification has altered the binding or toxic properties of the constituents;

2) To determine, in vitro, the selectivity and cytotoxic efficacy of these immunotoxins against tumor cells and the cellular constituents of the immune system;

3) To determine, in vivo, the selectivity and cytotoxic efficacy of these immunotoxins against tumor cells and the normal cellular constituents of the lymphatic system;

4) Using mitogens or allogeneic cells, to investigate the in vivo stimulation of the cellular constituents of the lymphatic system with special reference to the induction of the IL-2 receptor.

5) To use monoclonal antibodies in conjunction with complement to lyse selected target cells both in vitro and in vivo in the lymph nodes;

6) To use monoclonal antibodies coupled to radioisotopes for selective killing of tumor cells or normal cells in the lymphatic system;

7) To compare the pharmacokinetic behavior of immunotoxins with that of the native monoclonal antibody (see project # Z01 CB 08359-02 LTB);

8) To use the methods developed under objectives 1-7 to design rational chemotherapeutic modalities for the subcutaneous treatment of metastatic cells in the lymph nodes;

9) Similarly, to use the information obtained above to modify regional immune responses by selective ablation of lymph node cells.

Non-standard methods employed:

a) The preparation of antibody-toxin conjugates using the heterobifunctional reagent N-succinimidyl-3-(2-pyridyldithio)proprionate (SPDP).

b) Investigation of the effects of immunotoxins on cell viability using radiotracer techniques and fluorescent reagents.

c) Fluorescent immunochemical staining and flow cytofluorometric analysis.

d) Use of the SAAM modeling system to analyze the pharmacokinetics of antibody and immunotoxin distribution and binding in vivo.

Major findings:

1): Covalent coupling of ricin A-chain to monoclonal antibodies with the SPDP reagent has been obtained using 4 antibodies of different specificities. In each case, there has been little detectable loss of antibody affinity following the coupling reaction. In addition, the ability of the A-chain of ricin to inhibit protein synthesis at the ribosomal level is not significantly impaired.

2): Much of the initial work has been concerned with the monoclonal antibody D3 which recognizes the guinea pig hepatocarcinoma cell line L10. We have been unable to show any specific toxicity due to the D3-Ricin A-chain conjugate against L10 cells in vitro despite the fact that the immunotoxin binds to these cells with an affinity very similar to that of the native antibody and despite the fact that the conjugate is still able to inhibit protein synthesis in an acellular assay. Closer examination of L 10 cells showed that they lose some of the antigen recognised by D3 when they are removed from the animal and put into culture. In addition, fluorescence microscopy revealed that these cells are only poorly able to internalize either D3 alone or the D3-ricin A-chain conjugate. The antibody-antigen complex on these cells appear to be capped and then shed into the medium. Such activity could well account for the low reactivity of the D3-ricin A-chain immunotoxin.

3): Immunotoxins having specificities for mouse lymphocyte sub-populations have also been prepared. These reagents have been tested to determine their reactivities to target cells in vitro and have proved to be highly specific and toxic to the appropriate cell type. Drugs which are known to affect intracellular traffic (eg: ammonium chloride, nigericin) have also been used to augment the effects of these immunotoxins in vitro.

Some initial tests have been made in vivo to find out if the immunotoxins are able to kill specific cells or sub-sets of cells in the lymph nodes. We have not yet been able to show substantial cytotoxicity. However, experiments are currently being performed to investigate the kinetics of cell killing in order to optimize this system.

4): Experiments to determine the biological distribution of the uncoupled antibodies following administration via the lymphatic system are described in another report (Project # Z01 CB08359-02 LTB). These experiments must precede the in vivo use of immunotoxins and of antibody/complement.

Significance to Biomedical Research and to the Program of the Institute:

The objectives of this project relate to the possible therapeutic and diagnostic uses of monoclonal antibodies . This particular project may be considered as the chemotherapeutic arm of the on-going investigations of this group into the delivery of biological agents to the lymphatic system by subcutaneous injection. Previously we have demonstrated substantial targeting of monoclonal antibodies to the lymph nodes. This has been used to detect metastatic cells in the nodes of animals and has led to several clinical protocols. The present project is intended to follow up this work by producing cytotoxic systems directed to specific cell types within the nodes. In addition to the chemotherapeutic uses of these systems against cancer metastases, specific cell ablation in the lymph nodes may offer insights into the balance between regional and global control of the immune system and into the ways in which the immune responses may be usefully modified.

Proposed Course:

Each of the objectives proposed above will be pursued:

#1: We will investigate three separate questions which require clarification before immunotoxins can be usefully employed as chemotherapeutic agents in vivo.

a): The stability of the conjugates when in contact with the biological milieu;

b): The ability of these molecules to enter the lymphatic system and from there to penetrate into target tissues such as the interior of tumor masses or lymph nodes;

c): The selectivity and efficiency of these cytotoxic systems for their targets in vivo.

#2: Immunotoxins and immunoglobulin/complement techniques will be used for selective killing of metastatic tumor cells in the lymph nodes.

#3: Radiolabelled immunoglobulins will be used for selective killing of metastatic tumor cells in the lymph nodes.

#4: Analogous techniques will be used in attempts at selective ablation of immunologically significant cell subsets in the lymph nodes.

Publications:

Weinstein, J.N., Parker, R.J., Holton, O.D., III, Keenan, A.M., Covell, D.G., Black, C.D.V. and Sieber, S.M.: Lymphatic Delivery of Monoclonal Antibodies: Potential for Detection and Treatment of Lymph Node Metastases. Cancer Investigation, 3: 85-95, 1985.

Weinstein, J.N., Black, C.D.V., Keenan, A.M., Holton, O.D., III, Larson, S.M., Seiber, S.M., Covell, D.G., Carrasquillo, J., Barbet, J. and Parker, R.J.: Use of Monoclonal Antibodies for Detection of Lymph Nodes Metastases. in: Monoclonal Antibodies in Medicine (ICN-UCLA Symposium) in press.

Weinstein, J.N., Keenan, A.M., Holton, O.D.,III, Covell, D.G., Sieber, S.M., Black, C.D.V., Barbet, J., Talley, M.J. and Parker, R.J: Use of monoclonal antibodies to detect metastases of solid tumors in lymph nodes. in International Workshop on Monoclonal Antibodies and Breast Cancer (Symposium Volume) November 1984; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08368-02 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

A Mathematical Model of Subcutaneous Uptake of Monclonal Antibodies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David G. Covell, Ph.D., Senior Staff Fellow — LTB, NCI

Other Professional Personnel:

John N. Weinstein, M.D., Ph.D., Senior Investigator — LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.50	0.50	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Monoclonal antibodies or other ligands are potentially useful for the diagnosis and treatment of tumors. Their therapeutic and diagnostic potential depends on the ability of the antibody to reach the target cell.

We have developed a theoretical model for the biodistribution of whole monoclonal antibody and the FAB_2' and FAB' fragments into the lymphatic and capillary systems following intravenous and subcutaneous injection. The model incorporates processes for transcapillary and translymphatic solvent and solute movement that account for a) hydrostatic and osmotic pressure differences between the injected solution and fluid surrounding the injection site, b) differences in the available pore area for transport into the lymphatic and capillary systems and c) specific and nonspecific binding of antibody molecules to tissue cells at the injection site. The partial differential equations describing the model are being solved numerically on a VAX/11-780 computer.

Significant theoretical findings to date include the following: 1) most of the antibody that leaves the injection site to enter the lymphatics does so by convection in the fluid also entering the lymphatics, 2) most of the water leaving the injection site does so by entering the capillary system 3) intravenously administered antibody rapidly leaves the blood to enter major visceral organs and to a lesser extent skeletal muscle, skin and bone; (4) monoclonal antibody with specific target antigens in major organs receives greater than 75 percent of the administered dose; (5) the FAB fragment, when administered intravenously, demonstrates a much greater uptake relative to a nonspecific antibody over the whole antibody or over the FAB_2' fragment.

The concepts arising from this study are directly applicable to the design of clinical studies with monoclonal antibodies and other ligands.

Project Description

Objectives:

To investigate factors that improve the targeting of intravenously and subcutaneously administered monoclonal antibodies.

Nonstandard Methods Employed:

(1) Computer simulation of the partial differential equations describing the theoretical model using collocation methods; (2) simulation and data analysis using the SAAM and CONSAM compartmental modeling systems.

Major Findings:

(1) Most of the antibody that leaves the subcutaneous injection site to enter the lymphatics does so by convection in the fluid also entering the lymphatics; (2) Most of the water leaving the subcutaneous injection site does so by entering the capillary system; (3) Intravenously administered antibody rapidly leaves the blood to enter major visceral organs and to a lesser extent skeletal muscle, skin and bone; (4) Monoclonal antibody with specific target antigens in major organs receives greater than 75 percent of the administered dose; (5) The FAB fragment, when administered intravenously, demonstrates a much greater uptake relative to a nonspecific antibody over the whole antibody or over the FAB_2' fragment.

Significance to Biomedical Research and the Program of the Institute:

(1) Monoclonal antibodies or other ligands are potentially useful for the diagnosis and treatment of tumors in lymph nodes. Their therapeutic and diagnostic potential depends on the ability of the antibody to reach the target cell. (2) To improve delivery of monoclonal antibodies to target cells it is important to understand how molecules enter the lymphatic system.

Proposed Course:

(1) Continue theoretical studies on the movement of macromolecules from tissues into the capillary and lymphatic systems. (2) To apply the results of this analysis to a more complex model describing the distribution kinetics of monoclonal antibodies in nodal tissue. (3) To apply these models to the design and analysis of animal experiments and human trials.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08369-02 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

System Software for Protein and Nucleic Acid Structure Analysis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Lewis L. Lipkin, M.D.	Chief, Image Processing Section	LTB, NCI
Other Professional Personnel:		
Peter Lemkin, Ph.D.,	Computer Specialist	IPS, LTB, NCI
Bruce Shaipro, Ph.D.,	Computer Specialist	IPS, LTB, NCI
Morton Schultz,	Senior Engineer	IPS, LTB, NCI
Earl Smith,	Expert	IPS, LTB, NCI
Jake V. Maizel, Jr., Ph.D.,	Chief, Laboratory of Mathematical Biology	NCI
Ruth Nussinov, Ph.D.,	Visiting Scientist	LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Image Processing Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.8	0.

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

The objective is to functionally unite the wide variety of image processing equipment working in the laboratory so that the results of procedures performed on one processor may be available to the user at any other component. To this end two major software packages have been specified, designed, implemented and largely debugged. These are 1) BMIO, a basic set of input output routines which provide for interprocessor transfer of generalized digitized images, and 2) SPIDER a data independent context free packet switching network in which our DEC System 20 is the permanent master.

Project Description:

Objectives:

Our long term objective is to maximize the impact of our work on the protein analysis and nucleic acid structure research communities, and also to maximize the utility of the recently acquired hardware image processing systems (Comtal) in anticipation of the impending obsolesence of the PDP/8- quantimet predecessor. Accordingly we have devoted much time and effort to the problem of system export, first with GELLAB) the problem of production nucleic acid secondary structure calculation for gene sized sequences (a dedicated 68000 class processor(s) is essential) and lastly the construction of system software to allow control of all significant hardware via the System-20, the most "intelligent" of our processors. The system software involves construction of two components, BMIO and Spider, which are detailed below. The work on BMIO included the design specification, design document, programming, testing,and user document. The function of BMIO is to allow simple, flexible access to a variety of I/O devices (the RTPP's and disks) with data consisting of picture files in a multitude of data formats (1, 2, or 4 pixels per word), (picture, boundary, or other descriptive information), and picture sizes (256x256, 512x512, 256x4096, 4096x256, etc.). This work required the development of a user-controlled paging facility on the DECSYSTEM-2020. Once developed, many of the facilities of BMIO were made use of in other areas, such as the use of the paging system and of the disk access system in the nucleic acid secondary synthesis programs.

Methods Employed:

The function of SPIDER is to provide a simple, flexible mechanism for data-independent context-free packet switching among the DECSYSTEM-2020 and a variety of peripheral devices such as the COMTAL VISION ONE/20 image processing systems. An interface between the DECSYSTEM-2020 and the COMTALs is provided by a DEC PDP-11 used as a packet switcher in a modified wheel local network arrangement whereby the PDP-11 is the central hub of the network and all packets must go through it. However all devices hung off spokes of the wheel are not of equal status. The DECSYSTEM-2020 is always the master and whichever other devices are talking with it are always treated as slaves. In order to implement the network several things were needed. A device driver was needed for the PDP-11 to enable it to talk to the various devices attached to the network (the DECSYSTEM-2020 and the COMTALs). A set of packet encoder/decoder modules were needed for the PDP-11 to translate data and commands into a form suitable for the COMTALS. A network communications device handler was needed for the DECSYSTEM-2020 to enable it to talk to the PDP-11. A set of packet encoder/decoder modules were needed for the DECSYSTEM-2020 to translate data and commands from the user programs into a form suitable for the common network communications format. A set of test programs were needed to test and facilitate debugging of the implementation of the network.

Major Findings:

Implicit in the design of the packet encoder/decoder modules was a solid knowledge of the facilities provided by the COMTALs and how to gain access to them from a program residing on the PDP-11. In addition, in order to facilitate the implementation of the network although not absolutely necessary to the network we designed and coded a program residing on the DECSYSTEM-2020 which allows access to both the disk file system of the PDP-11 and its terminal system, including the control console terminal. This allowed the down-loading of programs from the DECSYSTEM-2020 to the PDP-11 and their transfer back to the 2020 for further development. It also provided for automatic bootstrap loading of the PDP-11 upon command from the 2020. This allows for true mastery of the network to be maintained in one central location, on the 2020. This program has since been modified to allow the 2020 to communicate with a variety of other computers, and is currently being used by Dr. Bruce Shapiro to communicate with the DEC VAX/750 in the laboratory of his close collaborator, Dr. Jacob Maizel of the Laboratory of Molecular Genetics in the National Institute of Child Health and Human Development.

Significance to Biomedical Research and the Program of the Institute:

It makes available to both on site and remote users within the Institute the complex of software and firmware facilities largely unique to the IPS. Thus image processing techniques can now be widely applied to the solution of problems of protein and nucleic acid structure and function.

Proposed Course:

With the computation and storage requirements for the future microGELLAB a dedicated microVAX/68000/16032 class processor is essential and work in the area of system analysis has progressed. Secondly, the construction of IPS SPIDER network software allows control of all significant hardware connected to our DECsystem-10 processor. Additional work on SPIDER now permits simultaneous device access as well as enhancements on SPITST (a maintenance software tool).

1. The midi C compiler was completed to the extent that it was usable enough to test, on our DEC10, concepts for the conversion of the GELLAB SAIL code to C. When nearly complete, we purchased a newly released C compiler for the DEC10 - the SARGASSO C compiler.

2. The major effort shifted to the conversion of the GELLAB SAIL code to C. PSAIL is a more complete SAIL to C translator than the original SAITOC program (started last year) and will hopefully enable us to export SAIL programs previously developed on our DEC10 to other processors of the VAX/68000/16032 class.

Work on this has been suspended indefinitely because of the departure during this fiscal year of Mr. Earl Smith. Prior to his departure, the work was brought to a level where the transmission of images among subsystems in the laboratory can be accomplished in an ad hoc manner. The extremely useful but quite complex programmed control of such interchanges in the context of the general Spider network will regrettably be deferred until such time as adequate manpower for the completion of its implementation can be obtained.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08370-02 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interactions in Globular Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert Jernigan, Ph.D. Theoretical Physical Chemist LTB, NCI

Other Professional Personnel:

Sanzo Miyazawa, Ph.D. Visiting Associate LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office. of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

Effective residue-residue interaction energies have been statistically derived from protein X-ray structures. A lattice-like model is used in which each residue type has a coordination number. If a specific residue has an incompletely filled coordination shell, then it is assumed to be filled with equivalent water molecules. Derived contact energies follow intuition: The most favorably interacting pairs are hydrophobic residues. However, those interactions are quite non-specific. More specificity is observed between polar residues. Protein folding schemes based on such energies favoring hydrophobic interactions are being developed.

GPO 914-918

Project Description:

Objectives:

The aim is to develop effective residue-residue interaction potentials derived on a statistical basis from protein X-ray structures. Solvent effects are included. These energies can be utilized to evaluate the "goodness" of various conformations.

Methods Employed:

Effective contact energies for proteins in solution were estimated from the numbers of residue-residue contacts observed in crystal structures on the basis of the quasi-chemical approximation with an approximate treatment of the effects of chain connectivity. A protein is regarded as a close-packed mixture of unconnected residues and effective solvent molecules whose size is the average size of a residue. The quasi-chemical approximation, that contact pair formation resembles a chemical reaction, is applied to this system with the basic assumption that the average characteristics of residue-residue contacts formed in a large number of protein crystal structures reflect actual differences of interactions among residues, as if contacts among residues and solvent molecules in each protein were in quasi-chemical equilibrium. The number of effective solvent molecules for each protein is chosen to yield the number of residue-residue contacts equal to its expected value at Flory's $\theta/T=0$ condition. A residue is represented by the center of its side chain atom positions, and contacting residues and effective solvent molecules are defined to be close pairs within a distance of 6.5A; nearest neighbor pairs along a chain are explicitly excluded in counting contacts. Coordination numbers, for each type of residue and solvent, are estimated and used to evaluate the numbers of residue-solvent and solvent-solvent contacts.

Major Findings:

Estimated contact energies have reasonable residue-type dependences, reflecting residue distributions in protein crystals; non-polar-in and polar-out are seen as well as the segregation between these residue groups. There is a linear relationship between the average contact energies for non-polar residues and their hydrophobicities reported by Nozaki and Tanford.

Significance to Biomedical Research and the Program of the Institute:

Development of a basic understanding of the folding of proteins into native states is essential to understanding cellular activities on a molecular basis.

Proposed Course:

The relevance of results to protein folding and several other applications of the present results are anticipated. These include locating hydrophobic nuclei for individual proteins and comparing these with known structures. Preliminary cases indicate these to be sensible. We intend to develop methods for generating

compact conformations of the most hydrophobic residues; from the limited number of such arrangements it should be possible to choose a few that are most likely to lead to a folded protein.

Publications:

Miyazawa, S. and Jernigan, R. L.: Estimation of Effective Interresidue Contact Energies from Protein Crystal Structures: Quasi-Chemical Approximation. Macromolecules 18, 534-552, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08371-02 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

B-Z Transitions in DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert Jernigan, Ph.D.,	Theoretical Physical Chemist	LTB, NCI
Akinori Sarai, Ph.D.,	Visiting Fellow	LTB, NCI
Sanzo Miyazawa, Ph.D.,	Visiting Associate	LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A simple model of the conformational transition between the right handed B double helix and the left handed Z double helix of DNA is proposed. This is a mechanistic model in which the dependence of the energy on twist is represented by two terms, one which depends upon the shape of the potential function through a series in powers of the twist and another inter-unit quadratic potential energy. This second term reflects the resistance of the DNA to deformations. With such a simple model we have studied cases of homogeneous chains with symmetric potential energies, as well as those within homogeneities and asymmetries in which one conformation is preferred over the other, for a portion of the chain. The method yields the location of the B-Z conformational boundaries for different conditions. Comparisons have been made with experiments in which G-C regions have been inserted in circular plasmid DNA. Additional methods have been developed to deal explicitly with the conformational statistics through this transition.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives:

Development of theoretical models to study conformational transitions between ordered and disordered states is relatively common. However, there has been little work in the area of transitions between two different ordered states. We are trying to develop simple methods to describe the B-Z transition to obtain information about the locations and extents of boundaries between the two conformations under different circumstances.

Methods Employed:

Transitions between B and Z forms in DNA have been studied with a model having a bistable energy function dependent on the twisting coordinate. Such a bistable system is expected to undergo a nonlinear structural transition upon twisting. We have made applications to the strain-induced transition in a circular DNA. The DNA is regarded as a linear chain of units with a double-well potential along the twisting coordinate, together with a harmonic potential for nearest-neighbor interactions. The latter serves to smooth distortions. We consider the total twist of the helix as the principal external variable and perform calculations for different amounts of twist. The minimization of total energy, with a Lagrange multiplier for the condition of fixed total twist, leads to a set of nonlinear equations which can be solved numerically. The mechanical model has not included statistical averaging over conformations. Methods to deal with such problems have been developed. It is important to include such effects since numerous configurations are accessible. The combinatory entropy may be important in the non-linear domain where positionally degenerate conformations are frequent.

Major Findings:

If a homogeneous uniform circular chain is twisted from its initial B form, the chain will be deformed uniformly, but when the twist exceeds a certain value a nonlinear solution appears, forming a Z region with two B-Z junctions. We have examined the B-Z transition behavior as a function of twist, together with further effects of an asymmetric potential and sequence inhomogeneities in which there are different preferences for the two conformations. Principal applications have been made to circular DNA with more labile inserted regions. We analyzed available experimental results, in which various lengths of alternating G-C sequence were inserted into a plasmid and conversions to Z DNA were observed upon changing linking number. This application was performed with the mechanistic model in order to estimate the magnitudes of model parameters. It was estimated that the energy barrier for the B-Z transition is of the order of 1 kcal/mole. From this analysis, if the length of the insert is less than a certain value, the entire insert converts to Z form at a critical linking number. The critical linking number increases as the length of insert increases, consistent with experimental observation, but decreases if the insertion exceeds a certain length. If the insertion is much longer, the B-Z transition exhibits a

different behavior, in which part of the insert flips to Z form and the Z part expands linearly upon changing linking number.

Significance to Biomedical Research and the Program of the Institute:

Conformational transitions are central to biological function. Developing methods to understand conformational transitions is important to a deeper molecular comprehension of biological processes.

Proposed Course:

We anticipate continuing studies of conformational transitions in both DNA and proteins.

Publications:

Sarai, A., and Jernigan, R. L.: A Model of the Strain-induced B-Z Transition. J. Biomol. Str. Dyn. 2: 767-784, 1985.

Miyazawa, S.: Statistical Mechanics of Supercoiling-induced B-to-Z Transitions in a Closed Circular DNA: One-Dimensional Model System with a Double Quadratic Displacement Potential and Long Range Interactions. J. Chem. Phys. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08372-02 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Interactions with DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert Jernigan, Ph.D.,	Theoretical Physical Chemist	LTB, NCI
Akinori Sarai, Ph.D.,	Visiting Fellow	LTB, NCI
Gene Barnett, Ph.D.,	Detail from ADAMHA	LTB, NCI
Percival D. McCormack, M.D., Ph.D.,	Senior Staff Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Ruth Nussinov, Institute of Molecular Medicine, Tel Aviv University Medical School

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Interactions of DNA with repressor proteins, drugs, carcinogens and free radicals are being studied. Shifts in electronic structures are being calculated with standard molecular orbital methods. The aim is to determine the dependence of the interactions on the DNA sequence and conformation.

Local sequence dependent models of conformations of DNA have been considered. Depending on the sequence, it is possible to logically construct both smooth bends and sharp bends in A/T rich regions of DNA.

PHS 6040 (Rev. 1/84)

GPO 914-919

Project Description:

Objectives:

Several studies of molecular interactions with DNA have been initiated. These include repressor-operator binding, drug and carcinogen binding and radiation effects, as well as local conformation models of DNA.

Various detailed models for DNA-protein binding are being investigated. These are being assessed for their likeliness by comparing calculated binding energies with experimental binding constants for native operator and mutant operators. Also calculations are performed for all sites in the entire species' DNA to determine the uniqueness of binding sites.

Structure-activity relationship (SAR) studies are underway to investigate DNA adduct formation with antitumor drugs and chemical carcinogens. Preferred binding sites and sites of covalent bond formation are sought. Likewise the sequence and conformational characteristics of both radiation susceptible and protected sites are sought.

Methods Employed:

Known models of repressor-DNA binding consist of a peptide helix fitted into the major groove of the DNA. The stability of such a model depends principally upon hydrogen bonding between the amino acid side chains and the base pair edges. Detailed models of several binding schemes are being investigated.

Quantum chemistry techniques are used via molecular orbitals to determine electronic alterations to DNA due to the adduct and to evaluate the strengths of various hydrogen bonds. For the SAR studies, cisplatin and a series of related analogs, where the site of interaction with guanine is thought to be intrastrand and covalent, is currently being studied. Planning is in progress for studies on polycylic aromatic hydrocarbons (PAH) where the site of covalent bond formation and characteristics of intercalation have been established for "ultimate" carcinogens formed by metabolic activation of the PAH. It is anticipated that alteration of the electronic densities of localized regions of DNA can be correlated to physical properties that will be amenable to experimental measurement. This will contribute to an improved understanding of the process of carcinogensis at the molecular level.

Local structure models of DNA depend on the interactions among local base pairs. We have included considerations of hydrophobic and electric interactions.

Major Findings:

We are testing various binding schemes for their uniqueness by calculating the binding energies for all sites within the species' DNA. With the conventional binding scheme it was found that in the entire lambda sequence, Cro binding to the lambda operator OR3 was uniquely best, although the strength of this preference is apparently related to only a few additional hydrogen bonds over the

number found at most sites in the non-specific binding mode. CI repressor binding is somewhat less specific and less strong; however the cooperativity of its binding serves to define an unique site by requiring neighboring binding sites at a specific separation.

Preliminary investigation of the effects of placing cisplatin near a guanine indicate that there is a significant effect on the electronic structures of atoms involved in the base pair hydrogen bonding.

In regions of A/T rich sequences it has been found that the DNA can assume sharp bends for some specific sequences and smooth bends for others. These conclusions have not yet been verified by appropriate experiments.

Significance to Biomedical Research ant the Program of the Institute:

Knowledge of electronic structure modifications in DNA from anti-cancer drugs and carcinogens may provide a better understanding of the chemistry of these processes. Understanding details of protein-DNA interactions is directly related to many basic areas of molecular biology.

Proposed Course:

Other binding schemes between the repressor and operator will be investigated. It is difficult to determine the uniqueness of such models, but testing against the experimental binding constants provides an extremely important test. Also, we intend to improve the details of the energy calculation by separately calculating relative strengths of hydrogen bonds.

Detailed calculations of the electronic structure as modified by placing the adducts at various positions will be continued. Also effects of DNA conformation on radiation susceptibility will be investigated by stiffening the DNA to various extents in an electric field.

Further studies of possible local interactions leading to sequence dependent local conformations will be pursued. Also, methylation can lead to further such conformation dependence on the methylation pattern.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08373-02 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Structure Function Relations in Nucleic Acids

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Charles DeLisi, Ph.D.	Chief, Theoretical Immunology Section	LTB, NCI

Other Professional Personnel:

Minoru Kanehisa, Ph.D.	Visiting Scientist	LTB, NCI
Kotoko Nakata, Ph.D.	Visiting Fellow	LTB, NCI
Peter Greif, M.D.	Staff Fellow	LTB, NCI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Theoretical Immunology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Methods are being developed to distinguish biological function sites from their spurious analogues in a given nucleotide sequence. We extended and applied the "perceptron" algorithm and discriminant analysis for distinguishing initiation and termination sites and intron/exon boundaries. Methods were developed that recognize specified patterns in nucleic acid sequences.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To develop statistical algorithms for reliably distinguishing sites or regions of biological interests in DNA. To characterize the frequency of occurrence of repetitive sequences in different regions of DNA.

Major Findings:

Protein coding regions and their boundaries exhibit significant patterns in DNA sequences such as consensus sequences of promoters and splice junctions, and the periodic appearance of specific bases reflecting the non-random usage of degenerate codons. The perceptron algorithm usually achieves 100% discrimination between correct and incorrect members after a learning procedure. However, its usefulness is limited when predicting unknown sequences, i.e. sequences not included in the learning procedure. Based on discriminant analysis, a more general method was developed to incorporate various features and to make better predictions. By this approach, the values for the perceptron matrices reflecting consensus sequence patterns at the boundaries, and the base composition and periodicity were combined. The method was tested by allocating sequences newly included in the database and shown to be very promising for predicting properties of the allocation of unknown sequences.

With respect to repetitive sequences we found that for longer repeat segments particular k-tuples sizes are favored, viz, 2, 4 and 5. The segments occur more frequently than would be expected of random sequences. In addition, the lengths of these repeat segments are greater than would be expected if the extension of the segments were solely attributable to a random process.

Significance to Biomedical Research and the Program of the Institute:

Animal genes, unlike those of most lower organisms are organized into introns and exons. Only the latter are translated. Therefore, computer algorithms which can predict the region to which a particular sequence belongs will be helpful for rapid translation of the growing body of DNA sequences.

The project potentially bears on the evolution of genetic organization in eukaryotes and its relation to cellular control mechanisms. Such mechanisms are expected to be important in both normal and pathologically functioning cells.

Proposed Course:

We intend to:

1) We will develop a program that combines the perceptron algorithm with all available statistical information for predicting the likelihood that a given nucleotide sequence is an exon intron or other coding region.

2) Model the non-random extension of the repeat segment vs. k-tuple size and explore its biological significance.

3) Examine the statistical variation in lengths and compositions of these repeat segments based on the DNA's classification, i.e. prokaryotic vs. eukaryotic, mammalian vs. bacterial.

4) Use the results from objectives 1 and 3 to help classify uncharacterized DNA.

Publications:

Nakata, K., Kanehisa, M., and DeLisi, C.: Prediction of splice junctions in mRNA sequences. Nucleic Acids Res., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08374-01 LTB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Membrane fusion: Structure, topology, and dynamics of tight and gap junctions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Pedro Pinto da Silva, Ph.D. Chief, Membrane Biology Section LTB, NCI

COOPERATING UNITS *(if any)*
Dr. J. Chevalier, Broussais Hosp., Paris (France); Dr. A. Aguas, Center Experim. Citol., Univ. of Oporto, Porto (Portugal); Dr. J. F. Moura-Nunes, Portuguese Cancer Inst., Lisbon; Dr. M.W.F., Ferreira, Inst. Hygiene and Tropical Medicine (Portugal)

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, FCRF, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.3	.3	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the spece provided.)*

We have studied the changes in membrane architecture that accompany the acrosome reaction, a secretory event of sperm cells that involves multiple fusions between plasma and acrosomal membranes of the sperm head. We found that the acrosome reaction starts (2-5 min after Ca^{2+} stimulation) with a dramatic redistribution of membrane proteins on the sperm surface. This lateral movement of membrane proteins is bimodal and leads to formation of three domains on the sperm plasma membrane that mimic the topology of the stable structural domains of the acrosomal membrane. Fusion and fission between the two membranes result in a tubular network of hybrid membranes that is arrested at the equatorial segment of the sperm head. The fenestrated veil of tubules that covers the sperm head is lost later on (5 min to 1 hr after Ca^{2+} stimulation); when the acrosomal reaction is completed the sperm head is limited by the inner acrosomal membrane. We continued the study of the junctional changes that accompany the reversal of the osmotic conditions in toad bladder epithelial cells. Water permeability studies carried out in Bourquet-type chambers showed that osmotic reversal did not damage the capacity of vasopressin to induce increases in the transepithelial transport of water. We have also shown hexagonal lattices (similar to gap junctions) proposed as morphological markers for rotavirus infections, in specimens taken from newborns. Therefore, these lattices cannot be used in the ultrastructure diagnosis of rotavirus infections.

PHS 6040 (Rev. 1/84) GPO 914-918

Z01 CB 08374-01 LTB
Formerly
Z01 CB 08282-02 LPP
Z01 CB 08268-03 LPP

Project Description:

Objectives:

1. To study the dynamics of membrane components during secretion. We have chosen the acrosome reaction as an elective model because the event can be triggered in vitro in a synchronous way and occurs in an extensive area of the sperm cell surface with the simultaneous formation of multiple sites of membrane fusion. In addition, we have recently developed a method to obtain total views of the sperm surface in freeze-fracture preparations. Our purpose is to address current questions on the dynamics of membranes during secretion: What is the role of lateral displacement of membrane proteins during secretion? What are the molecular mechanisms of membrane fusion? Do membranes of secretory vesicles modulate the structure of the plasmic membrane before fusion?

2. To study the structure, topology and mechanism of supramolecular assembly dynamics of tight junctions. To observe the capacity for transepithelial transport of water after treatment by vasopression.

Methods Employed:

1. Mature sperm cells from boars were incubated in Hanks' balanced salt solution supplemented with 10 µM glucose, during 1 hr at 37=C. The acrosome reaction was induced by the addition of 2 µM CaCl2 and 10 µM A23187 ionophore to the incubation medium. The reaction was arrested at 1, 5, and 10 min, and 1 hr by glutaraldehyde fixation (1.5%, pH 7.4). The fixed sperm cells were glycerinated, mounted in a double-replica device, and frozen in Freon 22 partially solidified by liquid nitrogen. The samples were freeze-fractured at $-140^{\circ}C$ and shadowed by platinum/carbon evaporation. The Pt/C replicas were cleaved, mounted in formvar coated grids, and observed by transmission electron microscopy.

2. For studies of tight junction proliferation in toad bladders, paired urinary bladders of Bufo marinus are mounted as sacs at the tip of a glass canula, according to the technique of Bentley. The serosal and mucosal faces of the tissue were bathed with an aerated amphibian Ringer solution (A6 Ringer containing 5 mM glucose). Osmotic shock was induced by immersing the hemi-bladders in distilled water for different periods of time (2, 5, 10, 30, 60 min) at room temperature or $37^{\circ}C$. In some cases, specimens were preincubated, before the application of the osmotic shock, with, in the serosal compartment, cycloheximide (200 µg/1, 30 min R.T. and 5 min $37^{\circ}C$) or cytoskeleton perturbers (colchicine 10^{-3} M, 2 hr R.T. followed by colchicine 10^{-3} + cytochalasin B µg/ml, 2 hr R.T.) or Ringer free Ca^{++}, free Mg^{++} + EGTA 2 µM (90 min R.T.). In other circumstances, after osmotic shock, the initial serosal (A6 Ringer + 5 mM glucose) was restored and the tissue incubated for various period of time (30 min to 3 hr). Epithelial cells were scraped, fixed in Ringer/glutaraldehyde 2% solution and processed for freeze-fracture.

Major Findings:

1. Our results are relevant to three areas of membrane biology: (a) The effect of Ca^{2+} and A2318 (a Ca^{2+} ionophore) on the structure of plasma and intracellular membranes; (b) the dynamics of membrane proteins during secretion; (c) the changes of membrane architecture that lead to the release of enzymes during the acrosome reaction, an event necessary for fertilization to occur. We found that: (a) Ca^{2+}, in presence of A23187, induces a rapid and dramatic redistribution of surface membrane proteins directed in two opposite poles of the sperm head surface; (b) the movement of surface proteins is probably guided by a putative interaction with the underlying acrosomal membrane; (c) multiple fusion between the two membranes leads to the formation of an exquisite labyrinth of tubles rather than, as previously thought, to the formation of isolated hybrid membranes.

2. We show that it is possible to assemble in vitro tight junctions. We show, for the first time a reversal of the polarity of the tight junction elements, that are now seen at the basal area of epithelial cells. We show also that basal cells by participating in the process of tight junction assembly can acquire a property that is characteristic of epithelial cells. We discovered the first physiologically valuable model capable of in vitro massive assembly of tight junctions. Physiological studies (J. Chevalier, J. Bourquet) showed that after reversal of the osmotic conditions the tissue is still capable of showing large increases in transepithelial transport of water upon induction by vasopressin. In these tissues we find the typical pentide aggregates that characterize the E faces of the apical membranes in tissues challenged by vasopressin or by cAMP.

Significance to Biomedical Research and the Program of the Institute:

The zonula occludens plays a major role in the structure and functions of epithelial tissues, binding cells together at their apical pole and sealing the inter-cellular spaces. Our proposal that inverted cylindrical micelles are principal elements of tight junction structure and that at the junctional site the exoplasmic halves of the plasma membrane are continuous is a radical departure from previous concepts, has important physiological implications, demonstrates the importance of nonbilayer lipid configurations in biological membranes and suggests numerous avenues of experimentation. Proliferation of tight junction strands is an interesting phenomenon that explains previous instances reported in studies of cellular pathology (the effect of ionizing radiations, for instance). The ability of osmotic shock to induce a massive proliferation of new junctional strands in the basal part of toad bladder epithelial cells, without affecting the apical zonula occludens, provides a useful system that is amenable to physiological study (e.g., water transport) and where the genesis and the dynamic as well as the composition and structure of tight junction strands can now be investigated. We have proposed that the zonula occludens represent a specialized instance of membrane fusion. The acrosome reaction represents a massive fusion event that can be induced experimentally.

Proposed Course:

We will continue to explore the structure, topology and mechanisms of supramolecular assembly of tight and gap junctions. In particular we will examine the influence of lipid disruptors on tight junction strands (in a previous paper we proposed that tight junction strands contain inverted cylindrical lipidic micelles). This proposal is now being tested in a number of laboratories within the U.S. and abroad. We will study the mechanisms of assembly of in vitro of tight and gap junctions, in particular the assembly of gap junctions upon action of cytoskelton perturbors. We will study the structure of both junctions by examination of stereo pairs at high magnification and in tight junctions, by computer stimulation of tight junction networks. In order to better characterize the membrane proteins that participate in the prefusion redistribution we found on the sperm surface, we want to use specific cytochemical markers (lectins, antibodies) coupled to colloidal gold. Methods of in situ, high-resolution, labeling of freeze-fractured cells, recently developed in this laboratory ("fracture-label" and "label-fracture") are to be used.

Publications:

Moura-Nunes, J.F., Ferreira, M.W.F., and Pinto da Silva, P.: Planar lattices in faeces of babies. Lancet, 8413: 1218-1219, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08375-01 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Fracture-Permeation: Molecular Spacing and Structure of the Cytoplasm

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Pedro Pinto da Silva, Ph.D. Cheif, Membrane Biology Section LTB, NCI

Other Professional Personnel:

Maria L.F. Barbosa, Ph.D. Senior Staff Fellow LTB, NCI

COOPERATING UNITS *(if any)*

Groupe de Recherches sur la Pathologid Renale (INSERM U.28; Professor J. Bariety, Broussais Hospital, Paris, France

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, FCRF, Frederick MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.3	.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We developed "Fracture-permeation" to investigate the intermolecular spaces of the cytoplasmic matrix. "Fracture-permeation" is the reversal of gel filtration: a macromolecule of known size and shape is used to assess the "mesh" of a chemically fixed cytoplasmic matrix. Cells and tissues are fixed in glutaraldehyde, impregnated in glycerol and frozen. The frozen specimen is freeze-fractured, thawed, and immersed in a concentrated solution of globular proteins. We use native ferritin (NF) and cationized ferritin (CF) to permeate model matrices of BSA, and to determine the compactness of the cytoplasmic matrix of (a) human lymphocytes and neutrophils; (b) isolated human lymphocytes activated by phytohaemagglutinin; (c) differentiation stages of the fungus Phytophtora palmivora and (d) striated muscle cells. With BSA matrices, we show that permeation depends on the diameter of the probe and on the intermolecular distances within the cross-linked matrix. Ferritin did not permeate the cytoplasm of mature human neutrophils, fungal zoospores and cysts, examples of cells with low levels of protein synthesis. In resting lymphocytes, permeation of ferritin was limited or absent; it became massive in cells activated by phytohaemagglutinin. Massive permeation of ferritin was observed within the cytoplasm of other active cells (sarcoplasm of striated muscle, fungal sporangia, germinating cysts). Therefore, compactness of the cytoplasmic matrix varies through differentiation and is related to the degree of cellular activity. In striated muscle, Fracture-permeation revealed distinct patterns of intermolecular spaces for each state of contraction. The patterns observed for sarcomeres in rigor, stretched or at relaxed states can be inferred from the "sliding filament model" (Huxley, 1969). In cardiac muscle, the contracted state (with penetration of the A band) suggests unexpected irregularities of molecular interactions during contraction. Our results validate "Fracture-Permeation" as a new method to investigate intermolecular distances in the cytoplasmic matrix.

Project Description

Objectives:

1. To develop a method to investigate the existence and distribution of intermolecular spaces within the cytoplasmic and extracellular matrices of glutaraldehyde-fixed cells. 2. To study the compactness of the cytoplasmic matrix at selected stages of cellular development. 3. To study the compactness of myofilaments within the sarcomere during muscle contraction. 4.

To study the chemistry of extracellular matrices in basement membranes.

Methods Employed:

We utilized an adaptation of the fracture-label technique ("fracture-permeation") to assess the compactness of the cytoplasmic matrix. Cells or tissues were fixed in glutaraldehyde, frozen and fractured in liquid nitrogen. After fracture, cell fragments were immersed in concentrated solutions of native ferritin (30% w/v). From the exclusion of these probes from the cross-linked cytoplasm, we inferred the existence and distribution of intermolecular spaces within the cytoplasmic matrix of glutaraldehyde-fixed cells. The cells and tissues utilized were: lymphocytes and neutrophils from peripheral blood, skeletal muscle from toad, cardiac papillary muscle from rat, and cells from different stages of morphological differentiation of the fungus Phytophthora palmivora. As a control, fractured cells or tissue were treated with Na-borohydride to reduce free aldehyde groups that could bind ferritin and prevent the free penetration of this electron-dense probe.

Major Findings:

We have developed a method--"FRACTURE-PERMEATION"--to investigate the existence and distribution of intermolecular spaces within the cytoplasmic matrix of glutaraldehyde-fixed cells. In essence, "Fracture-permeation" is the reversal of gel filtration: we use a macromolecule of known size and shape to assess the "mesh" of a chemically fixed cytoplasmic matrix. Cells and tissues were fixed in glutaraldehyde, impregnated with glycerol and frozen in liquid nitrogen. The frozen specimen was freeze-fractured, thawed, deglycerinated and immersed in concentrated solutions of globular proteins. We used native ferritin (NF) and cationized ferritin (CF) to permeate model matrices made of bovine serum albumin (BSA) and showed that permeation depends on the concentration of proteins within the cross-linked matrix: NF permeated matrices made from 10% or 15% (w/v) BSA solutions but did not permeate matrices made from solutions with 20% (w/v) protein or more. Despite binding to cross-fractured BSA matrices, CF was also able to permeate matrices made from a 10% (w/v) protein solution. We concluded that under experimental conditions that assure the solubility of a spherical Probe, permeation depends on the size of the probe and on the intermolecular distances in the cross-linked matrix. When NF does not permeate a cross-fractured matrix, the intermolecular distances in that matrix are smaller than 10 nm. When CF is unable to permeate the fixed matrix, it can be stated that the intermolecular distances within the matrix are smaller than three times

by ferritin. The dense cytoplasmic matrix of resting cells cannot be explained by models that envisage all cytoplasmic proteins congregated into microtrabcular lattices with the nature and dimensions proposed by Porter and co-workers. We offer a dialectic alternative to static structural concepts where change, rather than structure, defines the cytoplasm. In addition, marcomolecular permeation of freeze-fracture striated muscle characterized and distinguished rigor, contracted and relaxed states. Qualitatively distinct patterns of ferritin permeation into the sarcomere lead to immediate identification of all stages of muscle contraction. "Fracture-permeation" and the patterns of ferritin permeation of native ferritin revealed predicted qualitative changes in intermolecular spacing. These results validate "Fracture-permeation" as a method to investigate intermolecular distances in the cytoplasmic matrix.

Significance to Biomedical Research and the Program of the Institute:

"Fracture-permeation" makes possible the utilization of a new criteria, compactness of cytoplasmic and extracellular matrices, to the study of important processes as latency, cell growth, differentiation, transformation and aging. Fracture-permeation could eventually be used as a diagnostic tool to detect altered cytoplasm compactness in malignant cells.

"Fracture-permeation" permits to study the compactness of myofilaments within the sarcomere during muscle concentration and leads to immediate identification of all stages of muscle contraction. Fracture-permeation could be used to diganose disease of striated muscle. In kidney function, fracture-permeation can be used to approach, in human tissues, many problems that could only be approached in laboratory animals. We hope, within the next decade, to develop fracture-permeation in a routine clinical diagnostic tool.

Proposed Course:

The initial phase of this project was completed and submitted for publication. The technique which we discovered opens a new field of research: the study of the compactness of the cytoplasmic matrix. This can be pursued in a wide range of projects: from basic biology to the study of the cytoplasm in human disease, to its eventual use as a diagnostic tool (for instance, altered compactness in malignant cells or the diagnosis of the diseased striated muscle). In addition, the technique can, in principle, be modified to be used with fluorescent labels. These will make possible to use of "fracture-permeation" in rapid laboratory tests, an important requisite if diagnostic avenues are pursued. In the future, we will investigate the spacing limits within the cytoplasmic matrix using computer modeling.

In collaboration with researchers in the laboratory of Professor J. Bariety at Broussais Hospital in Paris, we have started to investigate the compactness of extracellular matrices, in particular the basement membrane in human glomeruli as well as the cytochemical study of the extracellular matrices in mesangial cells. We believe that by combining fracture-permeation and fracture-label, we can re-evaluate the physical, chemical, and immunological filtration barriers in glomeruli. As for both techniques use fixed, frozen tissue, human specimens can be collected, stored, and used throughout the study. At present, we are lacking the human resources to pursue this problem in our laboratory and, therefore, we must rely singly on our collaborators in France.

the diameter of CF, i.e., smaller than 30 nm. In gluaraldehyde-fixed cells, ferritin molecules were unable to permeate the cross-linked cytoplasm of mature human neutrophils, fungal zoospores and cysts, examples of nondividing cells with low levels of protein synthesis. In resting lymphocytes from human peripheral blood. permeation of ferritin was limited or absent, but it became massive in cells activated by phytohaemagglutin. Massive permeation of ferritin was also observed within the cytoplasmic matrix of other active cells (sarcoplasm or skeletal muscle, fungal sporangia, germinating cysts). We conclude that compactness of the cytoplasmic matrix depends on the physiological state of the cells: it varies through differentiation and is related to the degree of cellular activity. In nondividing cells with low levels of protein synthesis, the cytoplasm is not penetrated by ferritin. Therefore, the intermolecular distances within the glutaraldehyde-fixed cytomatrix of resting cells are smaller than 10 nm. These findings cannot be explained by models that envisage all cytoplasmic proteins congregated into "microtrabecular lattices" with the nature and dimensions previously proposed by Porter and co-workers. Our results accord with the existence in the native cytoplasm of interactive soluble and insoluble protein phases.

We showed that "FRACTURE-PERMEATION" can establish the distribution of intermolecular spaces in glutaraldehyde-fixed striated muscle. As examples of striated muscle, we used Sartorius muscle from toad Bufo marinus and papillary cardiac muscle from the left ventricle of Sprague-Dawley rats. A sequence of four distinct patters of ferritin distribution was associated to a progressive sequence of sarcomere lengths. The correspondence between sarcomere length and patterns of ferritin permeation permitted the recognition of sarcomeres in rigor (no penetration of ferritin), stretched (penetration into the I band and H zone) or at relaxed state (penetration into the I band only). Patterns of ferritin permeation were also recognized in oblique sections. In cardiac muscle, penetration of ferritin into A bands of contracted sarcomeres suggested unexpected irregularities in molecular interactions during contraction. Those irregularities may be magnified by glutaraldehyde fixation. Differences in distribution of ferritin were genuine, since random fractures gave ferritin direct access to all regions of the sarcomere. Patterns of ferritin permeation observed in sarcomeres in rigor, stretched or at relaxed state can be inferred from the changes in filament overlapping proposed by the "sliding filament theory" of muscle contraction. These results validate "Fracture-permeation" as a method to investigate intermolecular distances in the cytoplasmic matrix.

Technical Developments:

Establishment of an easy and convenient method to evaluate the compactness of cytomatices and to detect changes at selected cellular states.

Scientific Observations:

The compactness of the cytoplasmic matrix is related to the degree of cellular activity, varies through differentiation (P. palmivora), and characterizes different physiological states (muscle cells). Cells in a resting state had a crowded cytoplasm, impermeable to ferritin: growing cells were freely penetrated

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08376-01 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Fracture-label: Cytochemistry of freeze-fractured cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. Pinto da Silva	Chief, Membrane Biology	LMB(LTB), NCI
OTHER:	M.L.F. Barbosa	Sr. Staff Fellow	LMB, NCI/FCRF
	F.W.K. Kan	Visiting Fellow	LMB, NIC/FCRF

COOPERATING UNITS *(if any)*

Dr. J. Chevalier, U.28 Inserm, Hopital Broussais, Paris (France)
Dr. Ian Mather, Dept. Animal Sciences, University of Maryland, College Park

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI-FCRF, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.3	.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We developed a system of techniques--"fracture-label" cytochemistry--where fixed cells or tissues are freeze-fractured, thawed and labeled by conventional cytochemical methods. Labeling of both the protoplasmic and exoplasmic halves of fractured membranes can establish the composition of fractured membranes and the existence of specific transmembrane proteins. Fracture-label does not involve isolation and fractionation procedures: the membranes are fractured and marked in situ. In columnar, duodenal and goblet cells, we have shown that contrary to expectations, the membranes of the endoplasmic reticulum and the nuclear envelope contain numerous fucose residues labeled by Ulex Europaeus I (UEA). As expected, these membranes are not labeled by WGA. We conclude that subterminal implantation of sugars (fucose) does not prevent the backflow of glycoproteins. Numerous UEA receptors are labeled on P faces what shows that they are in part associated to transmembrane proteins. We have labeled cross-fractured nuclei and shown the association of glycoproteins to euchromatin. These results are the first qualitative, ultrastructural localization of glycoconjugates within the nuclear matrix. Fracture-label of human platelets from normal subsets as well as Bernard-Soulier and Glauzman Thrombasthenia shows that Con A and WGA receptors are associated with components that partition with the outer half of the membrane. In guinea pig mammary gland epithelia cells we show that butyrophilin, a glycoprotein of milk fat globule membranes, is present on the apical plasma membrane as well as on fat droplets inside the cytoplasm. Butyrophilin was detected by labeling with polyclonal antibody followed by protein A colloidal gold. In human neutrophils, choleragenoid/coloidal gold conjugates label E and P faces of the plasma membranes. Label on P faces may reflect the association of GM1 to a transmembrane complex.

Project Description:

Objectives:

1. To study the distribution and topology of various glyconjugates (i.e., mannose, fructose and sialic acid) in the duodenal columnar and goblet cells of small intestine. 2. To study the distribution of Con A and Ulex Europaeus I binding glycoconjugates in the euchromatin, heterochromatin as well as nucleolus in the nucleus of duodenal columnar and exocrine pancreatic cells. 3. To examine the partition and distribution of WGA and Con A receptors in plasma membranes of normal platelets and platelets from Glanzmann's thrombasthenia (GT) and Bernard-Soulier Syndrome (BSS). 4. To determine the distribution of selected glycoproteins on intracellular and on the plasma membrane of secretory mammary cells. To elucidate the routing of membrane proteins from intracellular sites to the apical surface of polarized cells. 5. To determine the partition and distribution of the glycolipid GM1 in plasma and intracellular membranes of human cells. To study the transport of the glycolipid GM1 from intracellular sites to the plasma membrane.

Methods Employed:

Cells or tissues were fixed in glutaraldehyde (1-25%) in phosphate buffered saline (PBS), impregnated in 30% glycerol and then frozen in liquid nitrogen cooled Freon 22 as described elsewhere.

Freeze fracture cytochemistry: Frozen tissues were freeze-fractured in liquid nitrogen with a scalpel. The freeze-fractured tissues were thawed (1% glutaraldehyde, 30% glycerol in PBS) deglycerinated in 1 mM glycylglycine or treated with Na-borohydride. Freeze-fractured cells or tissues were labeled and prepared for TS-FL or CPD-FL as described in previous reports.

Critical-point-drying: Fracture-labeled tissue blocks were osmicated in 1% osmium tetroxide in distilled water (30 min. room temperature), dehydrated through a series of graded alcohol and then critical-point-dried in a Polaron critical-point-drier.

Formation of platinum-carbon replica for electron microscopic examination: Fracture labeled and critical-point-dried tissue blocks were replicated by evaporation of platinum-carbon and carbon deposition. Upon removal of tissue debris by digestion in sodium hypochlorite, replicas were mounted on formvar-coated grids for electron microscopic examination.

Thin section fracture-label: Conventional methods are used to process fracture-labeled specimens for thin-section electron microscopy. The specimens are post-fixed in buffered 1% O_sO_4 (2 hr, 4°C). If necessary, membrane staining is enhanced by post-fixation in reduced O_sO_4 (addition of 1-2% potassium ferricyanide to the osmium solution, Goldfisher et al., 1981; Neiss, 1984). The samples are stained in bloc with uranyl acetate (0.5 mg/ml in veronal buffer, pH 6.0, 90 min), dehydrated in acetone or ethanol, and embedded in resin of choice. After resin polymerization, the blocks are trimmed, and thick sections are

inspected for selection of desirable fracture faces (see Fig. 1 in Pinto da Silva et al., 1981c). Because of the small size of fractured gel fragments relative to the original gel, cut faces are rare; cut faces are distinguished by their jagged outline.

Major Findings:

1. Comparative study of membrane glycocomponents in duodenum. Analysis of the distribution of WGA, Con A and Ulex europaeus I revealed different labeling intesity and patterns of the lectin binding sites in duodenal columnar and goblet cells. for example, Con A and Ulex europaeus I labeled strongly the E-face of the plasma membranes of duodenal columnar and goblet cells while WGA labeling intensity was only moderate. Con A label was absent in the mucous droplets of goblet cells. On the contrary, the number of WGA and UEA I binding sites in the mucous droplets was enormous. One finding which may be significant was that both Con A and WGA labeled only the E- fact of the microvillar and lateral cell membranes of columnar cells while UEA I labeled both E- and P-faces. UEA I label was also observed over the E and P faces of nuclear envelope membranes. Further experiments are needed to clarify and confirm these observations.

2. Cytochemical localization of glycocomponents in cross-fractured nuclei. We use fracture-label to establish ultrastructural localization of glycoproteins in cross-fractured nuclei of duodenal columnar and exocrine pancreatic cells. Mannose residues were detected in cell nuclei by labeling freeze fractured issues with Concanavalin A-horseradish peroxidase.colloidal gold (Con A-HRP.CG)[1] or direct Concanavalin A.colloidal gold (Con A.CG); fructose residues were detected with Ulex Europaeus I.colloidal gold (UEA I.CG) markers. Areas of the three main intranuclear compartments (euchromatin, heterochromatin, and nucleolus) exposed by freeze-fracture were determined by automated image analysis. Colloidal gold particles bound to each nuclear subcompartment were counted and the results expressed in number of colloidal gold particles per square micron ; S.E.M. Duodenal and pancreatic tissues fractured and labeled with Con A-HRP.CG complex or direct Con A.CG conjugates showed that the vast majority of Con A binding sites was confined to the euchromatin with only sparse labeling of the heterochromatin and nucleolus. UEA I labeling of duodenal columnar cells showed that colloidal gold particles were almost exclusively confined to cross-fractured euchromatin. Trypsinization of the fractured tissues prior to labeling with Con A and UEA I abolished 95-100% of the original label. Our results show that, within the nucleoplasm, mannose and fructose are residues of glycoproteins preferentially located in euchromatin.

3. In normal and pathologic platelets WGA and Con A binding sites were associated with membrane components that, upon fracture, partition with the outer half of the membrane. No WGA labeling was observed in neuraminidase treated cells or when succinylated-WGA was used instead of the native lectin. This shows that WGA binding is to terminal sialic acid and not to the internal GlcNAc or GalNAc residues of the surface glycoproteins (GPs). Densities of Con-A-Gold complexes/ug membrane contour appeared normal in GT platelets but significantly increased in BSS cells (8.5 ± 0.34, $n = 34$, vs. 4.59 ± 0.25, $n = 91$, $p(0.005)$. Beside the molecular defects reported on GT platelets, an alteration of the glycosylation of the other major GPs should occur in GT. In BSS, GPIb is absent but the general

and frozen thin sections, an approach of unusual difficulty with limited scope. Our "fracture-label" techniques circumvent these problems and permit--in an easy and straightforward manner--not only to locate a variety of membrane components but also to learn about their pattern of distribution and differential association with each membrane half. Previous knowledge of the distribution and topology of sialogly-conjugates in the plasma membrane and intracellular membranous organelles as well as secretory products of the duodenal columnar and goblet cells is vague. This knowledge is particularly lacking in the above two cell types with uranosyl-glycoconjugates. Our preliminary results with UEA I (a lectin specific for L- fructose residues) is particularly interesting. Results from these experiments would shed light on the presence of these glycoconjugates in the columnar and goblet cells of duodenum. They provide new information on the mechanisms that signal the sorting and traffic of membrane glycoproteins.

2. Cytochemical labeling of glycocomponents in cross-fractured nuclei. Our results represent the first attempt of a quantitative, cytochemical localization of glycoconjugates in the nucleoplasm of eukaryotic cells. We have also demonstrated that fracture-label can be used to detect glycoconjugates not only in freeze-fractured membrane halves of tissues and cells as demonstrated previously in our laboratory but also in cross-fractured nuclei. This has opened ways for in situ labeling at high resolution of nuclear proteins and antigens. At present, the role of nuclear glycoproteins remains unclear. Our findings of vast majority of Con A and UEA I binding sites in euchromatin may reflect an intimate and possibly an important relationship between glycoproteins and the replication and transcriptional activities that take place in this intranuclear compartment. Fracture-label appears to be a novel cytochemical approach that, combined to biochemical characterization, can be used to investigate the possible role of these nuclear glycoproteins. In the future, fracture-label can be applied to the investigation of glycoproteins and other nuclear antigens in cross-fractured nuclei of cancer cells and tumor cells as compared to that in normal cells.

3. Fracture label of normal, thrombasthenic and Bernard-Soulier platelets. In recent years, the application of highly sensitive biochemical techniques have lead to detailed descriptions of the composition of the platelet membranes. However, little is known on the molecular organization of these components within the plane of the membrane. The ability of "fracture-label" techniques to visualize both the inner core of the membrane and the cytochemical markers allows definition of the nature and the spatial arrangement of the platelet membrane components. This technique gives new insights on the data obtained in biochemical studies, e.g., with SDS-PAGE electrophoretic techniques, enzymatic degradation.

4. Localization of glycoproteins in membranes of secretory mammary cells. Throughout embryogenesis, the expression of specific surface domains is essential for the ordered formation of glands and tissues. In secretory epithelia the presence of regional differences in the plasma membrane ensures the regulated sorting and compartmentation of secretory products. Basic studies on the routing of apical plasma membrane proteins in lactating mammary cells have significance therefore to developmental and secretory processes.

GPs pattern remains normal and Con A binding sites are probably more accessible in BSS platelets than in normal cells. WGA-Gold complex densities were normal in GT and one BSS patient and reduced (36%, $p<0.005$) in a second BSS patient. Con A/WGA ratios were increased in the two BSS samples [1.72 and 1.05 vs. 0.59 (normal) and 0.48 GT]. This denotes strong modifications of the membrane organization of pathologic platelets with different expression according to the patient examined. In thrombin-aggregated platelets, Con A labeling still remained associated with the membrane outer half. In normal platelets, Con A binds preferentially to GPIIIa. This indicates that the association of GPIIb/IIIa complex with actin filaments which seems to occur during platelet aggregation is weak or might involve other membrane proteins loosely interconnected.

4. The localization of butyroplilin in secretory mammary cells represents the first successful use of antibodies in fracture-label techniques. As preliminary observations, we have confirmed that butyrophilin is located on the apical plasma membrane of secretory mammary cells: label was found on the E faces of the apical plasma membrane. Conversely, little or no label was seen on the P faces, an indication that butyrophilin is associated with the outer half of the apical plasma membrane. In similar samples, the nuclear envelope, endoplasmic reticulum and mitochondria remained unlabeled. Butyrophilin was also detected on apically located vesicles or on the surface of intracellular lipid droplets (the identification of this latter structure requires further use of TSFL). Intracellular fat droplets isolated by biochemical fractionation do not appear to contain butyrophilin. If butyrophilin is located on the surface of intracellular fat droplets, this glycoprotein, as found on FGM, does not necessarily arrive from the apical PM. However, this observation may provide clues as to the function of butyrophilin in the formation and secretion of lipid droplets from the apical surface.

5. Distribution and topology of GM1 in the membranes of human neutrophils. We have developed a conjugate of choleragenoid (subunit B of the cholera toxin molecule) and colloidal gold. The specificity of this conjugate to bind the glycolipid GM_1 is being tested. Besides the labeling of membrane halves, very little background label was obtained. Contrary to our expectations, we have observed labeling on both exoplasmic and protoplasmic halves of the plasma membrane. The unexpected labeling of protoplasmic membrane halves could suggest (a) the presence of GMI in the protoplasmic half of the membrane; (b) the association of GM1 to a transmembrane protein that partitions with the protoplasmic fracture-faces; or (c) could have been induced during preparation. We are now working to understand the significance of the label observed on protoplasmic membrane halves.

Significance to Biomedical Research and the Program of the Institute:

1. A comparative study of membrane glycocomponents in duodenum. Study of the mechanisms of glycosylation of membrane proteins is at present pursued in many laboratories, generally involving biochemical approaches. Because cell fractionation is necessary, cross contamination of membrane fractions makes it difficult to ascribe precise locations to glycosylated products. Cytochemical investigations rely on and labeling of autoradiography (with limited resolution)

5. Distribution and topology of GM1 in the membranes of human neutrophils. Glycolipids are ubiquitous components of biological membranes and can function as receptors, antigen determinants or as regulators of cellular behavior. Glycolipids are believed to reside in the exoplasmic half of membranes, a topology consistent with their physiological roles. Remarkable changes in glycolipid composition and metabolism are associated to cellular interaction, differentiation and oncogenic transformation. The binding of cholera toxin to a specific ganglioside (GM_1 receptor provides a model for the interaction of biologically active agents with glycolipids.

Proposed Course:

The experimental parts 1, 2 and 3 have been completed.

4. Localization of glycoproteins in membranes of secretory mammary cells. Mammary tissue will be processed through TS-FL to identify the cytoplasmic vesicles that bear the glycoprotein butyrophilin. We will also use monoclonal antibodies to different antigenic sites of butyrophilin to confirm the results obtained with the polyclonal antibody. We intend to extend our study to other FGM proteins.

5. Distribution and topology of GM1 in the membranes of human neutrophils. Because glycolipids are believed to reside in the exoplasmic half of cell membranes, we are working to understand the significance of the label observed on protoplasmic membrane halves. We are testing the specificity of our choleragenoid-colloidal gold conjugate and we will use antibodies against GM1 to check the authenticity of the label found on protoplasmic fracture-faces.

Publications:

Aguas, A. P., and Pinto da Silva, P.: High density of transmembrane glycoproteins on the flagellar surface of boar sperm cells. J. Cell. Biol. 99: 655-660, 1984.

Aguas, A. P., and Pinto da Silva, P.: The acrosome membrane of boar sperm: a Golgi derived membrane poor in glycoconjugates. J. Cell. Biol. 100: 528-535, 1985.

Chevalier, J., Pinto da Silva, P., and Caen, J.: Localization of WGA and Con A receptor sites on freeze-fractured human normal thrombosthemic and Bernard-Soulier platelets. J. Cell. Biol. 99: 179, 1984.

Pinto da Silva, P., Barbosa, M. L. F., and Kan, F. W. K.: Fracture-label: structure and cytochemical dissection of plasma and intracellular membrane halves; macromolecular permeation of the cytoplasmic matix; labelling of cross-fractured nucleoplasm. Proc. 8th Europ. Congress Electron Microscopy (Budapest), 1984, in press.

Pinto da Silva, P., Barbosa, M. L. F., and Aguas, A. P.: A guide to fracture-label: cytochemical labeling of freeze-fractured cells. In Koehler, I. K. (Ed.): Advanced Techniques in Biological Microscopy, Vol. III in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08377-01 LTB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: The Role of Myoglobin in Oxygen Transport

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David G. Covell, Ph.D. Senior Staff Fellow LTB, NCI

COOPERATING UNITS *(if any)*

Dr. John Jacquez, Department of Physiology & Biostatistics, University of Michigan, School of Medicine

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The role of hemoglobin in transporting oxygen in the circulation is clear; that of myoglobin in cardiac and red skeletal muscle is still open to experimentation and modeling. It has been thought to serve as a temporary oxygen store and to facilitate the delivery of oxygen by acting as a diffusive carrier. The idea that myoglobin facilitates the diffusion of O_2 in red muscle cells was a natural extrapolation from experiments on facilitated diffusion of O_2 across porous membranes containing concentrated solutions of myoglobin or hemoglobin. The experimental evidence for facilitated diffusion in muscle is not as clear and the contribution of theory less obvious, to a great extent because of a lack of concensus on the appropriate models to examine. Yet the problem is of profound importance for biology, not only for our understanding of oxidative metabolism in normal tissue but for out understanding of oxidative metabolism in tumor tissue. For mathematical modeling, it provides an example of a reaction-diffusion problem with non-uniform sinks and difficult boundary conditions. We have examined the role of myoglobin in facilitating oxygen diffusion across a 1-dimensional slab with a saturable consumption chosen to model mitochondrial consumption at the right boundary. Under these conditions myoglobin does not provide a large increase in oxygen transport for the range of PO_2's and diffusion lengths expected for skeletal muscle fibers. The next step will be to look at the pattern of O_2 consumption for a slab of 25-50 μm in thickness with bands of sinks at 5 μm intervals to simulate the structure of red muscle cells.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

1) To determine the role of myoglobin in facilitating oxygen delivery to tissue.
2) To establish conditions under which the nutrient supply of oxygen begins to limit the size of a tissue (or tumor) mass.

Methods Employed:

Model development is being done with the use of IMSL numerical software run on the VAX 11/780 computer system in the Laboratory of Mathematical biology.

Major Findings:

We have examined the role of myoglobin in facilitating oxygen diffusion across a 1-dimensional slab with a saturable consumption chosen to model mitochondrial consumption at the right boundary. Under these conditions myoglobin does not provide a large increase in oxygen transport for the range of PO_2's and diffusion lengths expected for skeletal muscle fibers.

Significance to Biomedical Research and the Program of the Institute:

A greater understanding of conditions where oxygen delivery via free or myoglobin-facilitated diffusion begins to limit the growth of tissue will be useful in the treatment of tumors.

Proposed Course:

The project is being continued with examination of more complex models.

Publications:

Jacquez, J.A. and Covell, D.G.: Models in the analysis of the role of myoglobin in the diffusion of oxygen in red skeletal muscle. Proceedings of the second international colloquium on Theoretical Biology and Medicine 1985, in press.

Covell, D.G., and Jacquez, J.A.: Does myoglobin contribute significantly to the diffusion of oxygen in red skeletal muscle. Am J. Physiology (Resp. Physiol.), 1985, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08378-01 LTB
Formerly
Z01 CB 08250-04 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Label-Fracture High Resolution Labeling of Cell Surfaces

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Pedro Pinto da Silva, Ph.D. Chief, Membrane Biology Section LTB, NCI

Other Professional Personnel:

Maria L. Barbosa, Ph.D. Senior Staff Fellow LTB, NCI
Frederick Kan, Ph.D. Visiting Fellow LTB, NCI

COOPERATING UNITS *(if any)* Dr. J. Chevalier, Broussais Hosp., Paris, France ; Dr. J. Boruget, C.E.E.N., Saclay, France; Dr. A. Macieria-Coelho, Dept. of Path. Cell., Inst. Rech. Cancer, Villejuif, France; Dr. M. Wilson, & A. Peratoni, LCC, NCI-FCRF; Dr. M.R. Torisi, Inst. Gen. Path. Univ. of Rome, Italy.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, FCRF, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.3	.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

"Label-fracture" allows the observation of cytochemical label on the cell surface. Cell surfaces labeled with colloidal gold are freeze-fractured and the fracture faces are replicated by Pt/C. The exoplasmic halves of the membrane are washed in distilled water. The new method reveals the surface distribution of the label coincident with the Pt/C replica of the exoplasmic fracture face. Initial applications indicate exceedingly high resolution (less than 5 nm) and low background. "Label-fracture" views of the distribution of the label on membrane surfaces while reserving cell shape and relating to the freeze-fracture morphology. Label-fracture is appropriate for routine use as a surface labeling technique.

Mapping of WGA and Con A receptors on the cell surface of boar sperm reveals intense WGA surface labeling over the region of the plasma membrane that overlies the acrosome. High density of WGA receptors is revealed over particle-free zone located at the base of the head. The density of WGA receptors in the tail is high over the mid-piece, annulus and principal piece. Intense labeling by Con A is observed over the sperm head with the exception of the particle-free zone at the base of the head where Con A receptors are absent. In the tail, Con A labeling is strong over the midpiece, absent over the annulus and sparse over the principal piece. In contrast to WGA, Con A receptors appear to co-distribute with the intermembrane particles. Our results confirm the exceptional promise of "label-fracture" to establish the surface distribution of antigens and receptors. Other label-fracture studies are underway: (1) apical surfaces of toad bladder epithelial cells; (2) localization and capping of cholera toxin receptors; (3) differences related to all aging and translocation; (4) capping of surface IgG in lymphocytes; (5) localization of insulin receptors on adipocytes.

GPO 914-918

Project Description:

Objectives:

To develop a method for high resolution of receptors and antigens over cell surfaces and to relate this distribution to the morphology of freeze-fractured plasma membranes. To investigate the supramolecular structure and dynamics of biological membranes.

Specific Aims:

1. Establishment of the experimental sequence in label-fracture. Interpretation of the new image provided by the technique. Adaptation to cells grown in monolayers and to the apical surfaces in transport epithelia.

2. High resolution cell surface labeling of wheat germ agglutinin and concanavalin A receptors in boar sperm by "label-fracture" cytochemistry.

3. Localization of cholera toxin receptors on neutrophils from human peripheral blood.

4. Movement of cell surface receptors and intramembrane particles during capping of surface IgG in human lymphocytes.

5. Topochemistry of the apical surface of toad bladder epithelial cells. Relation to E-face intramembrane particles and to vasopressin induced particle aggregates.

6. High resolution mapping of lectin receptor sites on the surface of cultured fibroblasts related to viral transplantation and aging.

7. Surface distribution of insulin receptors on rat adipocytes. .lm 1 .p 0,1

Methods Employed:

Cells are fixed in glutaraldehyde, labeled with lectin-protein-colloidal gold conjugates or other protein gold complexes, frozen and then freeze-fractured at $-130^{o}C$ by the double-replica method under high vacuum inside a Balzers freeze-etch unit. Formation of replica is competed by evaporation of Pt/C followed by carbon deposition.

Replicas are washed by successive floating on distilled water over a period of 2 to 3 hours. Replicas are mounted on formvar-coated grids for electron microscopic examination.

Major Findings:

1. The label-fracture technique is developed. We found that the outer half of plasma membranes (labeled by colloidal gold complexes) remains attached to the Pt/C replica of the freeze-fractured membrane. This allows the observation of

the distribution of a high resolution marker coincident with the freeze-fractured exoplasmic half of the membrane. We showed that there is no planar intermixing with components and that the resolution of the technique is probable under 5 nm. Label-fracture is now available for routine use.

2. We used "label-fracture" to establish a high resolution mapping of WGA and Con A receptor sites over the cell surface of boar spermatozoa and to investigate the possible association o the receptors to intramembrane particles. Label-fracture reveals intense WGA surface labeling over the region of the plasma membrane that overlies the acrosome including the equatorial segment. A decreasing labeling intensity is observed on the post-acrosomal area towards the posterior ring. This sparse labeling intensity changes abruptly to an unusually high density of WGA receptors over a sharply delimited particle-free zone located at the base of the head proximal to the cord area. The density of WGA receptors in the tail is high and homogeneous over the midpiece, annulus and principal piece. Narrow patches of rectilinear arrays of pits proximal to the annulus are not labeled. Intense labeling by Con A is observed coincident with the fractured E-face of the entire sperm head with the exception of the particle-free zone at the base of the head where Con A receptors are absent. The cord area is sparsely labeled. In the tail, Con A labeling is strong over the mid-piece, absent over the annulus and sparse over the principal piece. In contrast to WGA, Con A receptors appear to codistribute with the intramembrane particle revealed by freeze-fracture. Our results confirm the exceptional promise of "label-fracture" to establish with high resolution and sensitivity and surface distribution of antigens and receptors.

3. Preliminary experiments were done to examine the distribution of the glycosphingolipid GM1 on the plasma membrane of human neutrophils. To label the glycolipid, we used a conjugate of choleragenoid (subunit B of the cholera toxin molecule) and colloidal gold. We observed that although label was uniformly distributed on the surface of cells, the intensity of labeling varied among cells from the same population. This heterogeneity was also seen in thin-sections obtained from similar preparations.

4. We have used label-fracture to study the aggregation and "capping" of surface IgG on B lymphocytes isolated from human peripheral blood. In preliminary experiments, live B cells were incubated at room temperature with protein A conjugated to colloidal gold (protein A-CG). The incubation was terminated by addition of glutaraldehyde (to a final concentration of 2%) at zero, 5, and 60 minutes after mixture with the colloidal gold conjugate. We observed that the intensity of labeling varied among cells from the same population. Aggregation, "capping" and internalization of the protein A-CG label was seen after 5 minutes incubation with the label (internalization was followed with thin sections from the same cell preparations). On exoplasmic membrane halves, aggregation and capping of the label did not correspond to aggregation and/or capping of membrane particles. Gold label could be seen in areas devoid of membrane particles; this last observation suggests that surface IgG do not correspond to membrane particles see on exoplasmic fracturefaces. The analysis of complementary replicas will permit to correlate labeling to membrane particles on the protoplasmic half of the plasma membrane.

5. Label-fracture of the apical membrane of toad bladder epithelial cells shows that glycerol-induced aggregation of the intramembrane particles causes aggregation of WGA receptors at the cell surface. Initial studies indicate that the aggregates of intramembrane particles induced by vasopressin do not contain receptors of WGA.

6. Label-fracture localization of WGA receptors on the plasma membranes of human fibroblasts fails to reveal differences associated to aging or with viral transformation. In a single culture, a substantial heterogeneity of labeling intensity is observed which may be related to the cells cycle.

Significance to Biomedical Research and the Program of the Institute:

Label-fracture has been received with enthusiasm at scientific meetings. We have received numerous requests for inclusion of representative micrographs in forthcoming reviews and books, requests for review chapters and forthcoming manuscripts from other laboratories. It appears, therefore, that label-fracture will soon be used by many laboratories investigating the chemistry of cell surfaces. It is becoming increasingly clear that label-fracture has achieved the limits of resolution and sensitivity that are available by present day electron microscopic techniques and equipment. The study of the surface distribution of antigens and receptors on cell surfaces is not only important in the investigation of cell biological aspects of tranformation and malignancy; its applications are vast in many other domains including cellular endocrinology, neurobiology, microbiology and cellular immunology. We will pursue these objectives with enthusiasm.

Proposed Course:

The expansion of the label-fracture will proceed along two main lines:

1. The application and adaptation of label-fracture techniques to simple light microscopic methods with view to establishing routine laboratory tests in clinical practice.

2. To continue and expand the application of label-fracture in projects in course (as outlined) and in other lines of work as permitted by our resources as well as cooperative aggreements with other laboratories. Within the forthcoming year, the Section of Membrane Biology will proceed its activities in new laboratory space and facilities that will be almost ideal for the combination of cell biology/electron microscopy that characterizes our work. Expansion of our activity will, therefore, be proportional to the human resources that will be available.

Publications:

Pinto da Silva, P., and Kan, F.W.K.: Label-fracture: A method for high-resolution labeling of cell surfaces. J. Cell. Biol. 99: 1156-1161, 1984.

Bourguet, J., Chevalier, J., and Pinto da Silva, P.: Structure and surface cytochemistry of the luminal membrane in toad bladder epithelial cells. J. Cell Biol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08379-01 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Multicompartmental Analysis of Calcium Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David G. Covell, Ph.D. Senior Staff Fellow LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Alfred L. Yergey, NPM, NICHHD

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Abnormal calcium metabolism in adolescent children and postmenopausal women can have devastating consequences. The objective of this study is to elucidate the kinetics of calicum metabolism in normal children and to evaluate disease related changes in calcium metabolism in children and adults. Stable calcium isotopes were administered to children and women of childbearing age and serial samples were obtained for two to four days. Two stable isotopic tracers were used in these studies; one given orally and one given intravenously. The use of two tracers allows direct measurement of several important parameters of calcium metabolism, principally the fraction of calcium absorbed orally and the endogenous fecal excretion. Thermal ionization isotope ratio mass spectrometry was used to measure tracer enrichments in serum, urine, feces and food. The data was used to develop a multicompartmental model of calcium metabolism that better characterized calcium metabolic fluxes between regions of the body. The results of the initial studies showed the following; comparison of the data from two normal boys with a patient with fibrodysplasia ossificans progressivia (FOP) showed that the metabolic parameters of the normal boys were consistent with each other yet markedly differed from those of the FOP patient. The principal observations are that the fraction of dietary calcium absorbed is about the same for all three children, but that the FOP patient excretes virtually no urinary calcium. The mass of the compartment postulated for the non-skeletal internal calcium is about the same for the two normal boys and about the same size as the most rapidly turning over compartment in the FOP patient; the size of the remaining compartments for the model of the FOP data are 5-6 times greater than normal. Our observations are consistent with clinical observations and will contribute to improvements in the therapeutic treatment of FOP. Additional data is being collected for further testing of the proposed compartmental model. This research is being conducted in collaboration with Dr. A. Yergey of the Laboratory of Theoretical and Physical Biology.

GPO 914-918

Project Description:

Objectives:

To develop a multicompartmental model of calcium metabolism for populations that are inaccessible to studies using radiotracers; particularly in children and women of childbearing age.

Methods Employed:

Model development, simulations and optimizations are being done using software available in the IMSL statistical computing package and with the SAAM computing program.

Major Findings:

During the past year 3 normal boys, 3 normal prepubertal girls and one girl with fibrodysplasia ossificans progressivia (FOP) have been studied. Compartmental analysis of these data has shown that; 1) the plasma fractional catabolic rates and the average residence time of the calcium in the plasma compartment are in reasonable agreement with published data, 2) when compared with the data from normal children the FOP patient showed no malabsorption of dietary calcium, a significantly lower rate of urinary calcium excretion and a postulated greater fraction of total body calcium residing in a non-skeletal, non-plasma compartment. This latter finding is consistent the the clinical hypothesis that FOP is characterized by a large rapidly turning over soft-tissue calcium pool.

Significance to Biomedical Research and the Program of the Institute:

These studies represent the first studies of complete calcium metabolism in children older than infants. They are consistent with clinical observations in the case of FOP and as such they may contribute to an understanding of the progression of this disease.

Proposed Course:

The proposed course of this study is to increase the data base used to develop the multicompartmental model describing calcium kinetics. In addition to measuring calcium we will include measurements of hormones (PTH) and vitamin-D. These substances are integral participants in the control of calcium homeostasis and their roles will be included in the control portion of the compartmental model.

Publications:

Yergey, A.L., Covell, D.G., Hansen, J.W. and Vierira, N.E.: Calcium metabolism studied with stable isotopic tracers. Stable Isotopes in Nutrition, ACS Symposium Series 258, J.E. Turnland, P.E. Johnston, eds. Washingtion D.C., 1984, pp.27-37.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08380-01 LTB
FORMERLY
Z01 HD 00070-24 LMG

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Structure of Animal Viruses and Cells by Computational Analysis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jacob V. Maizel, Jr., Ph.D.	Chief, Laboratory of Mathematical Biology,	NCI

Other Professional Personnel:

Devjani Chatterjee, Ph.D.	Visiting Fellow	LTB, NCI
John Owens	Computer Specialist	LTB, NCI
Ruth Nussinov, Ph.D.	Visiting Scientist	LTB, NCI
Lewis Lipkin, M.D.	Chief, Image Processing Section	LTB, NCI
Bruce Shapiro, Ph.D.	Computer Specialist	LTB, NCI

COOPERATING UNITS *(if any)*
Dr. B. Fields, Dept. Microbiology and Medical Genetics, Harvard Medical School; Dr. D. Evans, NIBSC, London, UK
Dr. K. Currey, Dept. of Neurology, U. of Utah

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Picornaviruses cause diseases typified by polio, colds, hepatitis, and foot-and-mouth diseases. Sequences of these viruses have been examined for relationships among them and to other known and hypothetical proteins. Secondary structures of the RNAs have been found to vary with respect to pathological and sequences variances.

Adenoviruses are studied with a goal to understanding early events in virus replication wherein the cell's metabolism is subverted to viral functions, and late events during which assembly and morphogenesis occurs. Early viral proteins, whose existence was known from biochemical studies, have been analyzed by comparing their sequences to cellular proteins of known function. Physical association between the viral E3 glycoprotein and cellular MHC proteins correlates with homology between their sequences.

Techniques of biochemistry, virology, electron microscopy and computer analysis are used to study picornaviruses and adenoviruses. Analyses of proteins and nucleic acids have been developed and implemented. Graphic representations revealing homology, and reverse complementarity are coupled with numerical methods to aid the prediction of secondary structure, splicing, promoters, and recombination in nucleic acid molecules. Programs are developed and installed in a VAX 11/750 system designed for sequence analysis. Structures of up to 2000 bases have been predicted. Methods to assess the significance of predictions use Monte Carlo simulations, evolutionary comparisons and biochemical data. Protein secondary structure is being predicted from amino acid sequences. New sequences are compared with computerized databases to detect relationships with known proteins. Reovirus protein sigma-1 has been found to have a heptapeptide pattern typical of a coiled-coil, alpha-helical structure.

PHS 6040 (Rev. 1/84)

GPO 914-916

Project Description:

Objectives:

Our objective is to describe in molecular detail the components of animal viruses and cells with the aim of pinpointing the molecules that cause genetic and non-genetic changes in transformation and infection. The animal virus systems used as models have been poliovirus, adenovirus and murine oncornaviruses. A further objective is to develop techniques to characterize the pathology of mutational and cancerous changes at the level of molecular structure.

Methods Employed:

Representatives of the genomes of picorna-, adeno-, oncorna-, or other viruses and many cellular genes have been entirely sequenced. Previous work had provided a background of biochemical knowledge that can be correlated with analytical studies of sequence properties, and extended as ideas arise from these analyses. Patterns of bases in nucleic acids and in amino acid sequences derived from them are examined with a wide variety of searching algorithms using patterns that are designed by the investigator either ad hoc from pre-conceived consideration of molecular structure or revealed from mathematical or graphic pattern-detecting techniques. General principles of protein and nucleic structure are fitted to the predictions and to biochemical and biological observations. Fast, skimming methods are used to quickly find close relationships and more exhaustive homology and structure predictions are used to detect weakly similar structures and to extract maximum detail. Derived working databases are prepared and examined by relational database techniques to produce derived knowledge bases. Strong emphasis is placed on the comparative approach. Direct structure determination is given equal emphasis in an effort to read and interpret the information of the gene sequences.

Major Findings:

Picornaviruses: Secondary structure predictions of the genomes of virulent and attenuanted poliovirues types 1, 2, and 3, have shown structures near the 5' end of the genome that are conserved among strains. Bases that vary are concentrated in the unpaired secondary structure regions. This region is in the approximate 740 bases preceding the start of known protein encoding. In Sabin type 3 vaccination there is a rapid appearance of a variant strain that has increased neurovirulence. When sequenced these strains have a base change at position 472 (Evans, et al). RNA secondary structure is a candidate to explain this change in a non-coding region. Predictions by the program FOLD show increased formation of stems and loops around this position. It remains to be established whether this provocative result is characteristic. Comparisons of the genomes of a number of diverse members of this virus family show a strong similarity in molecular biology and structure by a wide variety of homology searches including the graphic matrix and Goad-Kanehisa local homology methods, yet show a wide variety of biology and tissue trophisms, including neurovirulence (Evans, et al) and, in the case of hepatitis A virus, liver (Baroudy, et al).

Adenoviruses: We have previously shown that in early stages of virus replication, before progeny DNA is made, a number of viral proteins can be detected by biochemical methods. When the sequences of these proteins, derived from the genome sequence, are compared with all other proteins in the Protein Identification Resource and GenBank(TM) public databases a significant homology is found with the human major histocompatibility protein (MHC), HLA-DR. This homology is especially intriguing because the virus protein was known for some time as being localized in the plasma membrane of infected cells, and more recently found to be isolable as a physical complex with the MHC using mild detergents, centrifugation and immune precipitation. This protein is not absolutely necessary for tissue culture replication. It is presumably important in natural infection because it is conserved among strains. Thus another instance of association between homologous proteins may be involved in the subtle interaction of virus with cells.

Collaborative studies on other viruses: In examining the proteins of reovirus type 3 (Bassel-Duby, et al) by Goad-Kanehisa homology and structure prediction a striking pattern of repeating, non-identical heptapeptide was found. The pattern has hydrophobic amino acids at positions 1 and 4 in the repeat segment and is strongly indicative of a coiled-coil alpha-helical structure. This protein is the sigma-1 hemagglutinin protein of the virus capsid. It is located at the vertices of the virion and is major factor in determining virus-host cell interactions.

Methodology: Expectations that simple pattern searches would detect constant, defined subsequences that would be unique for important features of the genome have not been realized. Yet, the information must be encoded in those sequences. Several algorithms were developed to search for patterns from more subtle viewpoints. In one approach the sequences are transformed using the Calladine-Dickerson rules for local variations in the helical structure of double-stranded DNA. Using this method (Nussinov, et al) DNAse sensitive sites in chromatin have been found to correlate with places in the sequence having large variations in the predicted base-pair roll and backbone torsion angles. A variation of this method in which periodic homologous variations in the twist-angle patterns were examined showed good correlation with sequences known to have enhancers even though direct sequence comparison could not detect a common pattern. Additional computer tools have been developed to allow rapid extraction and alignment of sequences from the GenBank database using any of the several dozen feature categories included. A variety of analyses can be performed on the aligned table. Work in progress suggests that non-random distributions of simple patterns occurs at levels that are not detectable by pairwise or three-way comparisons.

Significance to Biomedical Research and the Program of the Institute:

Structural characterization of the events in viral infections has defined critical features in cellular and viral regulatory mechanisms. Sequences of many of the most important pathogenic genes are accumulating rapidly, will continue to increase. New techniques using computers will be the only way to keep up in the analysis of this data. From those analyses we can expect to develop knowledge bases that will allow biomedical researchers and medical practitioners to

describe, understand and diagnose illnesses in much greater depth. This knowledge will aid in designing treatments that prevent or correct the damage caused by the pathologic genes of cancer, viruses and genetic disorders. Synthetic molecules may be designed to substitute, correct, or alter the pathogenic molecules produced by harmful genes.

Proposed Course:

We will continue to develop and apply techniques for revealing the information in nucleic acid and protein sequences. Special effort will be given to prediction of secondary structure of nucleic acids from their sequence and correlating it with biochemical data. Higher order sequence features associated with DNA structure will be examined from the point of view of interaction with other nucleic acid and protein molecules. New and refined algorithms will be implemented as we develop them or they are reported by others. If appropriate we will explore ways to use information from nucleic acid sequences to alter the replication of viruses, and modify the consequences to the cell.

Publications:

Baroudy, B.M., Ticehurst, J.R. Miele, T.A. Maizel, J.V. Jr., Purcell, R.H. and Feinstone, S.M.: Sequence analysis of hepatitis A virus cDNA coding for capsid proteins and RNA polymerase. Proc. Natl. Acad. Sci. USA 82: 2143-2147, 1985.

Chatterjee, D., Maizel, J.V. Jr.: Homology of adenoviral E3 glycoprotein with HLA-DR heavy chain. Proc. Natl. Acad. Sci. USA 81: 6039-6043, 1984.

Bassel-Duby, R., Jayasuriya, A. Chatterjee, D. Sonnenberg, N. Maizel, J.V. Jr., and Fields, B.N.: Sequence of reovirus haemagglutinin predicts a coiled-coil structure. Nature 315: 421-423, 1985.

Nussinov, R., Shapiro, B. Lipkin, L.E. and Maizel, J.V. Jr.: DNAase I hypersensitive sites may be correlated with genomic regions of large structural variation. J. Mol. Biol. 177: 591-607, 1984.

Nussinov, R., Shapiro, B. Lipkin, L.E. and Maizel, J.V. Jr.: Enhancer elements share local homologous twist-angle variations with a helical periodicity. Biochim. Biophy. Acta 783: 246-257, 1984.

Evans, D.M.A., Dunn, G. Minor, P.D. Schild, G.C. Cann, A.J. Stanway, G. Almond, J.W. Currey, K. and Maizel, J.V. Jr.: Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. Nature 314: 548-550, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00885-04 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Computer Aided Two-Dimensional Electrophoretic Gel Analysis (GELLAB)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Lewis L. Lipkin, M.D. Chief, Image Processing Section LTB, NCI

Other Professional Personnel:

Peter Lemkin, Ph.D.,	Computer Specialist	IPS, LTB, NCI
Morton Schultz	Senior Engineer	IPS, LTB, NCI
Earl Smith	Expert	IPS, LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Eric Lester, Univ. of Chicago, School of Medicine; Dr. Peter Sondreger, Univ. of Zurich; Dr. Richard Henneberry, Dr. Piotr Grojec, MNS, LMB, NINDS

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Image Processing Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

GELLAB is a computer based system of analysis of set of 2D gels. It incorporates sophisticated subsystems such as statistical, data base manipulation, image acquisition, etc. It has been applied to a variety of experimental systems in which quantitative changes in one or more proteins among hundreds or thousands of unaltered proteins is the basic analytic problem. During the year numerous extensions to the aramemtarium of procedures available to the user have been developed. It has also been applied to several new problems involving both early and late cellular differentiation and or protein synthesis. The objective of defining an exportable version of GELLAB (one that will run on a reasonably powerful microcomputer-affordable by a university department) is be actively pursued.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

The imperative of technology transfer argues for the further export of the GELLAB system (software and hardware specification) so that it is within the reach of individual university laboratories. In order to do this, it is being translated to the "portable" C language available on most other computer systems.

Methods Employed:

The translation will be performed using the PSAIL translator program currently being developed. The major thrust of the effort this year has been in developing the PSAIL SAIL to C translator. This is a major ongoing effort in automatic software to perform the SAIL to C conversion. As approximately 70,000 lines of SAIL code are involved in GELLAB, a completely manual conversion is unworkable. Current research with PSAIL indicates that close to 100% of the code can be converted automatically and futher extensions to GELLAB be performed in SAIL rather than C. Furthermore, other programs in this laboratory and other groups using SAIL could also be easily made portable when PSAIL is finished and when PSAIL itself runs on other types of computers. Additional work was done on the PSAIL design to insure portability and language extensibility and especially the ability for other PSAIL users to extend the language themselves.

Major Findings:

Additional collaborative work with Dr. Peter Sonderegger in Zurich has continued both in analyzing 2D gel data bases and in developing new gel analysis algorithms. The collaboration with Dr. Eric Lester at University of Tennessee Medical School on human leukemias has continued at a lower pace while the GELLAB conversion is underway. Additional evaluation of the Leukemia data base is continuing with a major effort planned in the coming months.

Enhancements to GELLAB

A major enhancement has been made to GELLAB during this past year. While working on the neuronal proteins project, a method for applying cluster analysis for groups of spot changes subjected to various experimental conditions was developed.

Significance to Biomedical Research and the Program of the Institute:

Alternation of the protein production of a cell as a result of drug or radiation effect is a fundamental manifestation of the changes associated with malignant transformation, etc., Indeed it seems likely that differences in patterns of protein production as revealed by GELLAB may serve to characterize subgroups of cells (eg.g. in leukemias) where morphology shows no difference.

Proposed Course:

The major collaboration with Dr. Peter Sonderegger (U. Zurich) is continuing.

The collaboration with Dr. Lester (U. Tenn.) is continuing with additional effort being placed on correlation of sequenced proteins and their 2D gel maps.

The collaboration with Dr. Richard Henneberry (MNS, LMB, IRP, NINDS) is continuing.

Publications:

Lester, E.P. and Lemkin, P.F., A GELLAB computer assisted 2D gel Analysis of states of differentiation in hematopoeitic cells. In Neuhoff, V. (Ed): Electrophoresis '84 Verlag Chemie, Basel, pp. 309-311, 1984.

Lemkin, P.F., Sonderegger, P., and Lipkin, L.E.: Identification of coordinate pairs of polypeptides: A technique for screening of putative precursor product pairs in 2D gels. Clinical Chemistry 30: 1965-1971, 1984.

Lemkin, P.F., and Lipkin, L.E.: 2D electrophoresis gel data base analysis: Aspects of data structures and search strategies in GELLAB, Electrophoresis 4: 71-81, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00886-04 LTB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chrerecters or less. Title must fit on one line between the borders.)*

Analysis and Synthesis of Nucleic Acid Secondary Structure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Lewis L. Lipkin, M.D.	Chief, Image Processing Section	LTB, NCI

Other Professional Personnel:

Bruce Shaipro, Ph.D.,	Computer Specialist	IPS, LTB, NCI
Morton Schultz,	Senior Engineer	IPS, LTB, NCI
Earl Smith,	Expert	IPS, LTB, NCI
Jake V. Maizel, Jr., Ph.D.,	Chief, Laboratory of Mathematical Biology	NCI
Ruth Nussinov, Ph.D.,	Visiting Scientist	LTB, NCI

COOPERATING UNITS *(if any)*

Dr. K. Currey, Molecular Structure Section, NICHD

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Image Processing Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Research has continued in collaboration with Dr. Ruth Nussinov from the School of Medicine at Tel Aviv University in the area of B-DNA distortions based upon the Calladine-Dickerson rules. Two extendable systems have been developed and enhanced which incorporate the four rules of distortion, namely, helical twist, base pair roll, torsion and propeller twist. These systems have the facility to search for patterns that are not known to exist a priori. Feature enhancement and feature detection techniques are available to aide the user in an interactive environment to discover new patterns of potential interest. These techniques have indicated the existence of morphologic structures which appear to be correlated to functionality within the molecule. Work has also begun on a new algorithm to measure similarity among secondary structures of RNA molecules. This technique when perfected should permit the determination of multiple levels of similarity amongst several molecules of the same or different classes. Also, the RNA secondary structure drawing program has been sent to several more institutions around the world. It is proving to be quite useful in depicting the structures of RNA molecules after they have been folded.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

The understanding of the structure of nucleic acids and how it relates to a molecule's functionality is a main objective of this project.

Methods Employed:

Two systems have been developed with the collaboration of Dr. Ruth Nussinov, a visiting scientist from the Institute for Molecular Medicine at Tel Aviv University with the object to provide a researcher with a set of tools for locating features that might be of significance and which were not known to exist in advance.

One system deals with sequences on an individual bases containing over 30 functions for manipulating and displaying the results of the manipulations on the four rules described above. The system has proven quite useful for determining patterns in sequences, that were not known a priori, as a function of the Calladine-Dickerson paradigm.

Major Findings:

The second system has incorporated some of the ideas inherent in the first system into a two-dimensional sequence comparison system. Here, the results of applying the Calladine-Dickerson rules may be viewed as a similarity comparison using several sequences at one time. Using some special features inherent in the Comtal image processing computer, we have been able to overlay and enhance patterns that would have been very difficult to discover by other means. Certain symmetrical structures have been discovered in areas near mRNA start sites based upon these techniques. About 30 functions are available that allow the manipulation of dot matrices for comparison. Among these is a new algorithm which speeds up the processing of diagonals within such matrices.

Significance to Biomedical Research and the Program of the Institute:

The project is directed toward the basic structural correlates of nucleic acid function. As such it is fundamental to such Institute concerns as carcinogenesis, mutagenesis, gene expression, etc.

Proposed Course:

Work has begun on a new algorithm to measure similarity among secondary structures of RNA molecules. This technique when perfected, should permit the determination of multiple levels of similarity amongst several molecules of the same or different classes. Included in this research has been the exploration of various LISP systems, e.g., Rutlisp, Franz Lisp, Xlisp and Loglisp, for the implementation of such algorithms and other algorithms that require the manipulation of complex data structures.

The RNA secondary structure drawing program has been sent to several more institutions around the world to aide in the research of nucleic acid secondary structure.

Publications:

Nussinov, R., Shapiro, B., Lipkin, L.E. and Maizel, J.: DNAase I hypersensitive sites may be correlated with genomic regions of large structural variation. J. Molec. Biol. 177: 591-607, 1984.

Nussinov, R., Shapiro, B., Lipkin, L.E. and Maizel, J.: Enhancer elements share local homologous twist angle variations with helical periodicity. Biochimica Biophysica Acta, 783: 246-257, 1984.

SUMMARY STATEMENT

ANNUAL REPORT

LABORATORY OF CELLULAR ONCOLOGY

DCBD, NCI

October 1, 1984 through September 30, 1985

The laboratory of Cellular Oncology plans and conducts fundamental research on the cellular and molecular basis of neoplasia. Investigators develop and employ tissue culture cell systems and animal models to study the induction and maintenance of benign and malignant neoplasia and reversal of the neoplastic state, elucidate structure-function correlations through detailed examination of individual genes which have been implicated in neoplasia, and examine spontaneous tumors from humans and other species for the presence of exogenous genes or altered cellular genes. The main research results for the past year are as follows:

Tumor virus expression in vitro and in vivo:

This project studies mechanisms by which tumor viruses or cellular genes contribute to oncogenesis and seeks to devise approaches to prevent or reverse such changes in cells. The major focus has been on the p21 ras oncogenes and papillomaviruses. We have studied various ras oncogenes and the ras containing Harvey murine sarcoma virus (Ha-MuSV). Yeast and mammalian ras genes have been shown to be functionally equivalent, since each can function in the heterologous system. These results suggest that mutational studies of ras gene function in yeast, which are much easier to carry out than in mammalian cells, may be relevant to the function of ras genes in mammalian cells. The ras oncogene has also been used to show that an activated oncogene may induce metastatic potential in some cells, but not in others. These results suggest that onc gene activation may represent a critical genetic determinant of metastatic potential; however, other factors also contribute to the cell's metastatic potential. Introduction of Ha-MuSV DNA into a human breast cancer cell line renders these cells tumorigenic for nude mice that have not received estrogen, although the parental cell line requires exogenous estrogen to be tumorigenic. These results suggest that onc gene activation may be one mechanism by which estrogen independence occurs clinically. Site directed mutagenesis has identified a cysteine residue near the C-terminus of all ras genes that is absolutely required for the transforming function of the protein, its membrane localization, and its binding of lipid. Variant Ha-MuSV that carry only one of the two point mutations present in the wild type Ha-MuSV are oncogenic, but not as oncogenic as the wild type virus. Non-coding sequences upstream and downstream from the ras coding sequences have a dramatic influence on the oncogenic activity of the virus. These results contribute to our understanding of the mechanisms by which ras transforms cells. Papillomavirus research has been basic and clinical. Using frame shift and deletion mutagenesis, we have identified two genes in Bovine papillomavirus that can independently transform established mouse tissue culture cells. The protein product of one of these transforming genes has also been identified in transformed cells; this represents the first non-structural papillomavirus protein to be identified. Its biochemical properties can now be studied. This gene may be involved in human and animal tumors associated with papillomaviruses. Detection of the homologous protein in human papillomavirus infections may prove to be useful diagnostically. A family with epidermodysplasia verruciformis (EV; wide-spread, chronic papillomavirus infection) has

been identified in which EV is inherited as an X-linked recessive trait. Since familial EV usually displays an autosomal recessive inheritance pattern, our findings suggest that lesions in different chromosomes can lead to EV.

Regulation of retroviral replication and cellular oncogene expressions

The long range purpose of this project is to investigate the roles of cellualr oncogenes and retroviruses in neoplasia and to use viral mutants for elucidating regulatory mechanisms of gene expression associated with cell differentiation and oncogenesis. Oncogene amplification, rearrangement, and expression has been studied in several different malignant tumor cell lines. Two human teratorcarcinoma cell lines were found to have 3- to 4-fold amplification and rearrangement of the Ki-ras-2 genes. There was also a 4-fold increase in k-ras mRNA. NIH 3T3 cells transfected with DNA from these cell lines showed transformed foci and produced tumors in nude mice. Amplification and increased expression of the fos oncogene was noted in a choriocarcinoma cell line, and increased fos and N-ras mRNA was found in a human hepatoma cell line. In a model system that employed an anchorage dependent mink cell line non-productively infected with Moloney sarcoma virus (which contains the v-mos oncogene), superinfection with a novel dualtropic mouse retrovirus induced anchorage independent cell growth that correlated with a marked amplification and increased expression of the v-mos oncogene.

Genetic mechanism of carcinogenesis and biological modifiers as defense mechanism

The major goals of this project are to elucidate the molecular genetics of neoplastic transformation of normal tissues and the purification and function of biological modifiers that may be important to host defense. A transforming gene has been detected in a human hepatocarcinoma cell line. The transforming activity of this gene can be significantly enhanced by treatment of the cell DNA with aflatoxin (AF) B-1-epoxide, which is a potent carcinogen. A 3.1 kb DNA fragment has been molecularly cloned from the genomic DNA of the cell line. The fragment is currently being sequenced to determine if it shares any homology with known oncognes. Preliminary data suggest that the fragment contains sequences related to retroviral regulatory elements (long terminal repeats). The clone displays a low constitutive level of transforming activity on NIH 3T3 cells. Treatment of the DNA with AFB-1-epoxide results in a more than 300-fold increase in transforming activity. Further studies of this clone and its interaction with AFB-1-epoxide may yield significant insights into carcinogenesis by this class of carcinogen. In biological modifier studies, a lymphokine called cytotoxic cell differentiation factor (CCDF) has been studied functionally and purified. This lymphokine has been isolated from unstimulated murine peritoneal macrophages. CDCC is required in conjunction with IL-2 to form lymphokine-induced cytotoxic cells (LICC), which are thought to represent an important host defense mechanism against neoplastic cells. The induction of LICC requires IL-2 followed by CDCC. Based on their cell surface markers, the LICC induced by these factors represent a unique class of cytotoxic cells. The extent of involvement of this lymphokine system in surveillance against neoplastic cell and the anti-tumor activity in vivo of these LICC are currently under study.

Biological studies of various normal, virus-infected, and malignant cells

The primary purpose of this project is to study some of the pertinent factors which influence cell differentiation and malignant transformation. A major current emphasis is to define more precisely the mechanisms by which interferon (IFN) acts on cells. The test system used is the virus-negative methylcholanthrene induced BALB/c sarcoma, Meth A, which can be passed both in vivo and in vitro. In this system, comparison of the response in cell culture with that in the intact mouse makes it possible to analyze the direct action of IFN on Meth A cells separately from the host defenses. Cells treated in vitro were analyzed for the anti-cellular effects in vitro and immune reactivity in vivo. At the concentrations tested, IFN was cytostatic, but not cytotoxic. Meth A cells treated with alpha-beta IFN were rejected more efficiently than those treated with gamma IFN. The ability of immunodeficient mice to support the growth of IFN treated Meth A cells was compared with their growth properties in immunocompetent mice. Immunodeficient mice included nude mice as well as mice rendered immunocompromised by treatment with either cyclophosphamide or cyclosporin A. In each instance, the IFN treated cells grew much better in the immunodeficient animals. These experiments confirm the necessity of functional T cells in order for IFN to exert its antitumor effect. The results obtained with IFN treatment of Meth A suggest that the major effect of IFN on chemically induced sarcomas is mediated through the host immune response, rather than by its anti-cellular activities.

Murine leukemia viruses of various host range and disease potential have been studied biochemically. Various tissue culture cell lines have been developed. Using one of these cell lines, a new group of murine leukemia virus has been identified and characterized. Several murine leukemia viruses have been molecularly cloned as infectious DNA molecules. Using these molecules, work is in progress to determine the biochemical nature of various biological functions.

Studies of several groups of murine leukemia viruses and their relationship to many inbred strains of mice have contributed significantly to our understanding of many viral diseases. Recombination between sequences of replicating virus and host cell sequences in the generation of new virus is already well known. Isolation of additional groups of viruses is dependent on the use of cell culture systems in which the viruses can be replicated. Using a Mus dunni cell line, several new viruses which were previously undetected have been isolated. Biological and biochemical characterization of some of these new viruses and their role in pathogenicity may prove useful to our understanding of disease production.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03663-09 LCO

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Tumor virus expression in vitro and in vivo

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Douglas R. Lowy, Chief, Laboratory of Cellular Oncology, NCI
Other: Elliot J. Androphy, Medical Staff Fellow, LCO, NCI
Ricardo A. Feldman, Senior Staff Fellow, LCO, NCI
John T. Schiller, Guest Researcher, LCO, NCI
Pierre E. Tambourin, Visiting Scientist, LCO, NCI
Nancy L. Hubbert, Microbiologist, LCO, NCI
Alexander G. Papageorge, Microbiologist, LCO, NCI
William C. Vass, Biologist, LCO, NCI

COOPERATING UNITS *(if any)*
Laboratory of Pathology, NCI, Drs. R. Muschel and L. Liotta
Medicine Branch, NCI, Drs. A. Kasid and M. Lippman
University Microbiology Institute, Copenhagen, Denmark, Dr. B. Willumsen

LAB/BRANCH
Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.5	5.0	2.5

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors B/D
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project seeks to study mechanisms by which tumor viruses or cellular genes contribute to oncogenesis and to devise approaches to prevent or reverse such changes in cells.

Experiments with oncogenes have used various ras oncogenes and the ras containing Harvey sarcoma virus (Ha-MuSV). Yeast and mammalian ras genes have been shown to be functionally equivalent, since each can function in the heterologous system. Site directed mutagenesis has identified a cysteine residue near the C-terminus of all ras genes that is absolutely required for the transforming function of the protein, its membrane localization, and its binding of lipid. Variant Ha-MuSV that carry only one of the two point mutations present in the wild type Ha-MuSV are oncogenic, but not as oncogenic as the wild type virus. Non-coding sequences upstream and downstream from the ras coding sequences have a dramatic influence on the oncogenic activity of the virus.

Papillomavirus research has been basic and clinical. Using frame shift and deletion mutagenesis, we have identified two genes in Bovine papillomavirus that can independently transform established mouse tissue culture cells. The protein product of one of these transforming genes has also been identified in transformed cells; this represents the first non-structural papillomavirus protein to be identified. A family with epidermodysplasia verruciformis (EV; widespread, chronic papillomavirus infection) has been identified in which EV is inherited as an X-linked recessive trait. Since familial EV usually displays an autosomal recessive inheritance pattern, our findings suggest that lesions in different chromosomes can lead to EV.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

1. To improve the biologic assay of DNA-mediated gene transfer (transfection).

2. To determine the effect of a variety of physical and chemical treatments on the biological activity of specific DNAs (viral and cellular).

3. To gain insight into how the state of differentiation of a cell regulates virus and gene expression.

4. To define the portions of viral genomes which induce cellular transformation.

5. To study the structure, oncogenic potential and control of normal gene sequences which are homologous to the oncogenes of transforming retroviruses.

6. To analyze the normal function of genes homologous to the oncogenes of transforming retroviruses.

7. To study spontaneous human tumors for their expression of oncogenes and for the ability of tumor DNA to induce cell transformation.

8. To study the cellular origin of retrovirus components.

9. To study the evolution of retroviruses.

10. To study viral recombination, especially as it relates to the development of sarcoma viruses, leukemia viruses, and papillomaviruses.

11. To evaluate the effects of hormones and other chemicals on endogenous and exogenous virus expression.

12. To propagate and study papillomaviruses in tissue culture.

13. To define the functional organization of papillomavirus genomes especially with regard to their transforming and oncogenic activity.

14. To develop assays for papillomavirus proteins.

15. To measure the relatedness of viruses in warts from different patients and species, including those from patients with epidermodysplasia verruciformis (EV), laryngeal papillomas, condyloma acuminata, flat warts, common warts, plantar warts, and warts associated with immunodeficiency.

16. To screen skin cancers of EV, kerato-acanthomas, and other tumors for papillomaviruses or tumor genes.

17. To test reagents for their anti-tumor or anti-viral activity in vitro and in vivo.

Methods Employed:

1. Treatment of cells or animals with hormones, other chemicals, and tumor viruses.

2. Detection of retrovirus expression biologically by XC plaque test and focus induction.

3. Specific radioimmune, fluorescence, and peroxidase techniques are used for antigen detection in whole cells, extracts of whole cells, and fractionated cell components.

4. For the isolation of genomic DNA, DNA is extracted from tissue culture cells or tumors by the Marmur technique, except that proteinase K is used instead of pronase, since higher molecular weight DNA is obtained by this modification. Unintegrated viral DNAs are enriched by the Hirt procedure.

5. Transfection of DNA utilizes the calcium phosphate technique of Graham and Van der Eb as modified by Stow and Wilkie. Cell or viral DNAs are assayed for biologic activity in the DNA transfection assay. This activity is then correlated with the expression of the transfected genes in the cells.

6. Virus is grown in sensitive tissue culture cells and purified by sucrose density centrifugation in a zonal rotor. Isotopically labeled single stranded viral DNA probes are synthesized in an endogenous reverse transcriptase reaction carried out in the presence of antinomycin D or in exogenous reactions following purification of viral RNA on sucrose density gradients.

7. Nucleic acid hybridization between specific probes and cell or viral nucleic acids is carried out in liquid or by the Southern blotting technique.

8. Cellular and viral genes are molecularly cloned and amplified in prokaryotic systems. The cloned DNAs are then used as probes for molecular hybridization and for structure-function studies.

9. Specific deletions, mutations, or recombinations are introduced in the cloned DNAs to map biological and biochemical functions.

Major Findings:

1. Significant progress has been made in viral and cellular oncogene studies. Our principal model system has been the Harvey murine sarcoma virus (Ha-MuSV), whose p21 ras transforming protein differs from its normal cellular ras counterpart at only two amino acids (12 and 59). One series of experiments seeks to determine the role of Ha-MuSV ras protein coding sequences as well as non-coding sequences in determining the

oncogenic activity of the virus. Variant Ha-MuSV viruses that contain a point mutation only in amino acid 12 or 59 are more oncogenic than an isogenic virus that encodes the normal version of the protein. However, the wild type virus (with both mutations) is more oncogenic than either virus that contains only one of the mutations. Deletion of certain 3' noncoding viral sequences lowers the transforming activity of the virus and changes its target cell. This lowered oncogenicity is correlarted with lower viral p21 levels. Preliminary analysis suggests that the viral sequences contain an enhancer element in this region. These results indicate that ras genes with single point mutations can be highly oncogenic, that both mutations in the virus are additive, and that minor changes in the virus can alter its target cell.

These recombinant viral genes are also being used in collaboration with the Laboratory of Pathology (R. Muschel and L. Liotta) to study the possible involvement of oncogenes in tumor metastasis. NIH 3T3 cells morphologically transformed by either the normal ras gene or by mutated versions of the ras gene are tumorigenic for nude mice. However, only those NIH 3T3 cells transformed by the mutated versions of the gene form metastases. Another cell line transformed by these genes also forms tumors locally, but it does not form metastases. These results indicate that an activated oncogene may have metastatic potential in some cells, but not in others. In collaboration with the Medicine Branch (A. Kasid and M. Lippmann), NCI, the consequences of introducing Ha-MuSV DNA into a human breast cancer cell line (MCF-7) have been studied. Although the parental cells require exogenous estrogen to be tumorigenic for nude mice, introduction of the HaMuSV DNA renders the cells tumorigenic without estrogen.

Another set of experiments has explored the role of sequences near the carboxy terminus of the Ha-MuSV p21 protein. In collaboration with the University Microbiology Institute, Denmark (B. Willumsen), we have established mutation of a cysteine residue that is present in all ras genes near the C-terminus leads to a mutant p21 that is deficient for cellular transformation, the tight binding of lipid to p21, and migration of the protein to the plasma membrane. Although the majority of the ras protein coding sequences are similar in different ras genes, the 20 amino acids immediately upstream from this required cysteine residue are highly divergent among different ras genes. We have determined that deletion and duplication of these sequences has little influence on the transforming activity of the gene. These results indicate that this portion of the protein is not essential to the transforming function of ras genes.

In collaboration with investigators at Merck Sharpe and Dohme (D. deFeo-Jones and E. Scolnick), we have found that modified yeast ras genes can induce tumorigenic transformation of mammalian cells. Mammalian cells were also shown to substitute functionally for yeast ras genes in yeast.

2. Interesting results have also been obtained with papillomaviruses (PV). We have extended our molecular genetic studies of bovine PV induced cellular transformation. We have previously localized the transforming region to a 69% viral DNA fragment. We have now provided genetic evidence

that BPV contains at least two genes that can independently transform an established mouse cell line. One gene coincides with the E6 open reading frame (ORF) located near the 5' end of the 69% transforming fragment. The other is located within the E5 ORF at the 3' end of this fragment. These genes can be activated by placing a retroviral long terminal repeat (LTR) upstream from either transforming region. The E5 gene can transform mouse C127 and NIH 3T3 cells, while the E6 gene transforms C127 cells, but not NIH 3T3. We have also identified the E6 protein product in cells transformed by the E6 gene. This is the first non-structural papillomavirus encoded protein that has been detected.

We have also made observations in Epidermodysplasia Verruciformis (EV), a disease of chronic widespread warts which can undergo malignant changes. We have found a pedigree with EV in whom susceptibility to widespread papillomavirus infection is inherited as an X-linked recessive trait. An autosomal recessive inheritance pattern has been reported previously in familial cases of EV.

Signifigance for Biomedical Research and the Program of the National Cancer Institute:

The p21 ras genes are a multigene family. The reasons underlying their multiplicity may be important both for understanding their normal function and their possible role in specific cancers. Activated forms and amplified numbers of these genes have been found in many animal and human tumors. Therefore their mechanism of action may be relevant to the pathogenesis of many tumors. The activated cellular ras genes have differed from the viral ras genes by containing only one point mutation, while the viral ras genes contain at least two point mutations. Our studies with the ras-H gene of Ha-MuSV indicate that ras genes with single point mutations can be oncogenic in vivo. These results strengthen the hypothesis that such genetic changes, which have now been found in many human tumors, may play a significant pathogenetic role in these tumors. Our observation that changes in the non-p21 coding sequences of Ha-MuSV can alter the target cell of the virus correlates with the finding of activated cellular ras genes in many different types of tumors.

Finding that the C-terminus of the viral p21 protein is required for its biological activity and its migration to the cell membrane suggests that the protein must reach the plasma membrane in order to carry out its physiological or pathological roles.The critical cysteine at the C-terminus that helps mediate these functions is encoded by all ras genes, which implies that these results are directly relevant to the function of the human ras genes. The sequences directly upstream from the required cysteine apparently function principally as a hinge to link the membrane anchoring domain at the C-terminus to the catalytic domain at the N-terminus.

The ability of yeast and mammalian ras genes to function in their heterologous systems implies that mechanistic results obtained with ras genes and their products in one species will be relevant to all ras genes. Since mutational studies are easier to carry out in yeast than in mammalian cells, these results suggest that yeast ras gene function may be relevant to the function of mammalian ras genes.

The development of estrogen independence obtained with the human breast cancer cells containing an activated ras gene suggest oncogene activation as one mechanism by which such changes occur clinically. Similarly onc gene activation may represent a critical genetic determinant of metastatic potential.

Papillomaviruses are a common cause of benign epithelial tumors in humans and other species. Some lesions induced by these viruses undergo malignant conversion. Patients with epidermodysplaia verruciformis induced by human papillomavirus (HPV) type 8 and certain other HPV types have developed many cutaneous maligancies in association with their skin lesions; these malignant tumors contain HPV DNA. The inheritance of EV is usually autosomal recessive in familial cases. Our finding a family with a strikingly different inheritance pattern suggests that lesions in different genes can lead to the EV phenotype.

Recent studies have found HPV DNA sequences in approximately 80% of human cervical cancers and in many premalignant cervical lesions. Little has been known about the functional organization of the papillomavirus genomes or how lesions progress from a benign to a malignant state. The determination of the transforming sequences of BPV DNA represents a potentially important step towards understanding how these tumors are formed. Our current studies are the first to detect a virus that carries two genes either of which can transform established cell lines. The BPV E6 protein is the first non-structural papillomavirus encoded protein to be identified. The E6 proteins encoded by other papillomaviruses and other non-structural papillomavirus proteins may be identified by a strategy similar to the one we have employed here. Human cervical carcinoma cell lines that contain HPV DNA appear to retain and express E6 sequences selectively, suggesting that this gene may be significant for the oncogenic properties of these cells. Detection of the protein permits its biochemical properties to be studied. The identification of non-structural papillomavirus proteins may also prove useful diagnostically; dysplastic and malignant lesions that harbor PV genomes do not usually express structural PV proteins, but they may express non-structural genes.

Proposed Course:

The work is in progress.

Publications:

Nakabayashi, Y., Dvoretzky, I., Chattopadhyay, S.K., and Lowy, D.R.: In vitro transformation by bovine papillomavirus. J. Invest. Derm., 83: 12s-17s, 1984.

Willumsen, B.M., Christiansen, A., Hubbert, N.L., Papageorge, A.G., and Lowy, D.R.: The p21 ras C-terminus is required for transformation and membrane association, Nature, 310: 583-586, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09050-01 LCO

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on endogenous murine leukemia viruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S. K. Chattopadhyay, Visiting Scientist, LCO, NCI

Other: M. R. Lander, Microbiologist, LCO, NCI

COOPERATING UNITS *(if any)*

M. W. Cloyd, Duke University Medical Center, Durham, NC
H. C. Morse III, Chief, Laboratory of Immunopathology, NIAID
J. W. Hartley, Head, Section on Viral Oncology, NIAID

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Murine leukemia viruses of various host range and disease potential have been studied biochemically. Various tissue culture cell lines have been developed. Using one of these cell lines, a new group of murine leukemia virus has been identified and characterized. Several murine leukemia viruses have been molecularly cloned as infectious DNA molecules. Using these molecules, work is in progress to determine the biochemical nature of various biological functions.

Studies of several groups of murine leukemia viruses and their relationship to many inbred strains of mice have contributed significantly to our understanding of many viral diseases. Recombination between sequences of replicating virus and host cell sequences in the generation of new virus is already well known. Isolation of additional groups of viruses is dependent on the use of cell culture systems in which the viruses can be replicated. Using a Mus dunni cell line, several new viruses which were previously undetected have been isolated. Biological and biochemical characterization of some of these new viruses and their role in pathogenicity may prove useful to our understanding of disease production.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

The major goal of our studies is to biologically and biochemically characterize various groups of endogenous murine leukemia viruses to understand their biological nature and evolution.

Methods Employed:

The current projects require various tissue cultural techniques, biological assay of various murine leukemia viruses, DNA transfection, isolation of high molecular weight DNAs, isolation of RNAs, isolation and purification of unintegrated proviral DNAs by Hirt's method, restriction endonuclease. Analysis of viral genomes, agarose gell electrophoresis, Southern and Northern blotting, nick-translation of DNA, molecular hybridization, molecular cloning, and DNA sequencing.

Major Findings:

1. Development of a cell line for the propagation of various groups of murine leukemia viruses. A Mus dunni cell line has been developed that is permissive for all four classes of murine leukemia viruses (MuLV): ecotropic, amphotropic, xenotropic, and mink cell focus-forming viruses. The M. dunni cells contain fewer MuLV-related sequences than do feral or domestic mouse, rat, or mink cells. Infection of the line by ecotropic MuLV induces a distinct cytopathic effect, and the cells can be readily transfected by MuLV DNA. The M. dunni line has been used to isolate an endogenous MuLV from the SC-1 feral mouse cell line.

2. A new type of retrovirus present in many murine leukemia systems. A new type of murine leukemia virus has been detected and isolated from normal and leukemic AKR and C58 mice, as well as from NFS mice inoculated with Friend or Moloney ecotropic viruses. These new viruses are XC negative, serologically cross-react with MCF env antigens, but are ecotropic in host range, being able to infect mouse cells to varying degrees and unable to infect mink or other cells infectable by MCF or xenotropic viruses. Such viruses from AKR cross-interfere with Moloney MuLV and MCF viruses in SC-1 cells and appear to have similar properties to the SL3-2 isolate. Analysis of their genomes by restriction endonuclease mapping of proviral DNA demonstrated structures similar to class II MCFs with 5' half of the genome similar to ecotropic virus and the env region possessing restriction sites characteristic of MCF viruses. In normal AKR mice, ERV (ecotropic recombinant virus) is found in spleen and bone marrow as early as one week of age, and appears at 3-4 months in thymus. ERVs have not been detected in mice with no or low expression of ecotropic viruses (NFS, NZB, DBA/2, BALF/c, C57BL/6). Because of their apparent recombinant structure and ecotropic host range, we have provisionally designated them as ERV to distinguish them from the MCF class of MuLV.

Significance to Biomedical Research and the Program of the Institute:

Studies of several groups of murine leukemia viruses and their relationship to many inbred strains of mice have contributed significantly to our understanding of many viral diseases. Recombination between sequences of replicating virus and host cell sequences in the generation of new virus is already well known. Isolation of additional groups of viruses is dependent on the use of cell culture systems in which the viruses can be replicated. Using a Mus dunni cell line, several new viruses which were previously undetected have been isolated. Biological and biochemical characterization of some of these new viruses and their role in pathogenicity may prove useful to our understanding of disease production.

Proposed Course of Research:

The work is in progress.

Publications:

Lander, M. R., and Chattopadhyay, S. K.: A Mus dunni cell line that lacks sequences closely related to endogenous murine leukemia viruses and can be infected by ecotropic, amphotropic, Xenotropic, and mink cell focus-forming viruses. Journal of Virology 52: 695-698, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05550-16 LCO

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of retroviral replication and cellular oncogene expressions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Kenneth S. S. Chang, Medical Officer, LCO, NCI

Other: Lai-che Wang, Visiting Fellow, LCO, NCI

COOPERATING UNITS *(if any)*
VA Hospital, Washington, DC
Department of Preventive Medicine, Public Health Service, Washington, DC
Laboratory of Molecular Oncology, NCI

LAB/BRANCH
Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	2.0	0.3

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☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The long range purpose of this project is to investigate the roles of cellular oncogenes and retroviruses in neoplasia, and to use viral mutants for elucidating the mechanism of regulation of gene expression associated with cell differentiation and oncogenesis.

The topics of current interest are: 1) Oncogene rearrangement, amplification, and expression in human hepatocellular carcinomas, teratocarcinomas, choriocarcinomas, murine reticulum cell neoplasms, and trophoblastic tumors. Two human teratocarcinoma cell lines were found to have 3- to 4-fold amplification and rearrangement of Ki-ras-2 gene. There was also a 4-fold increase in the mRNA which hybridized with v-Ki-ras probe. NIH/3T3 cells transfected with DNA from these cell lines showed transformed foci and produced tumors in nude mice. An amplified c-fos and its enhanced expression was noted for a choriocarcinoma cell line, and an increased production of mRNA for c-fos and c-N-ras was noted for a human hepatoma cell line. 2) Roles of mos and other ocogene expression in the anchorage-independent growth of S+L- mink cells superinfected with a novel murine retrovirus exhibiting dual-tropic (B- and xenotropic) properties. Marked amplification and increased expression of mos appear to correlate with the anchorage-independent growth of the superinfected S+L- mink cells. 3) Regulation of retroviral replication in murine trophoblasic tumor cells. The non-permissiveness of trophoblast cells was found to be dominant in the hybrid cells formed by fusion with a cell line permissive for retrovirus replication. 4) Screening for monoclonal antibodies and human antibodies against HTLV. Hybridomas have been screened and some positive ones were obtained. A sero-epidemiologic survey for human anti-HTLV antibodies has been completed. 5) Permissiveness of nonlymphoid cells to HTLV-I. Some cell lines produce syncytial cells after HTLV-I infection, but the HTLV genome could not be shown to be present after months of in vitro culture.

Project Description

Objectives:

The objectives of this project are: (1) to examine human hepatomas, teratocarcinomas, choriocarcinomas, murine reticulum cell neoplasms, and trophoblastic tumors for possible amplification, rearrangement and enhanced expression of cellular oncogenes; (2) to define the roles played by c-mos and other oncogene expression in the anchorage-independent growth of S+L- mink cells superinfected with a novel dual-tropic retrovirus; (3) to study the regulation of virus replication in murine trophoblastic cell lines; (4) to screen human sera for HTLV antibodies and to produce monoclonal antibody against HTLV antigens; and (5) to test various nonlymphoid cell lines for their ability to support replication or express mRNA and viral antigens of HTLV.

Methods Employed:

High molecular weight DNA extracted from normal as well as tumor cell lines was treated with restriction enzymes before gel electrophoresis and blot-hybridization with P-32 labeled oncogene probes prepared from cloned recombinant DNA. After autoradiography, the patterns of hybridization bands were compared among various cells. The expression of cellular and viral mRNA was detected by quick-blot hybridization with appropiate probes. DNA transfection of NIH/3T3 and C127 cells was performed by calcium phosphate precipitation method and the cells were observed for transformed foci and inoculated into nude mice to test their tumorigenicity.

Major Findings:

1. Oncogene rearrangement, amplification, and expression in human hepatomas, teratocarcinomas, choriocarcinomas, murine reticulum cell neoplasms and trophoblastic tumors. These human tumor cells are of special interest because of their difference in developmental cell lineages representing endodermal, embryonic, and extraembryonic origin, respectively. Murine reticulum cell neoplams and trophoblastic tumor cells are also of special interest because of their B cell and extraembryonic cell lineages, respectively.

It was found that two human teratocarcinoma cell lines, Tera-1 and Tera-2, showed evidence of amplification and rearrangement of Ki-ras-2 gene. The degree of amplification was determined by serial dilution with appropriate internal controls, such as measurement of c-mos or c-sis which are not amplified, and found to be 3- to 4-fold. However, only certain portions of c-Ki-ras-2 gene showed amplification as evidenced by differences in the pattern and intensity of hybridization among different fragments produced by various restriction endonuclease cleavage. The cellular mRNA which hybridized with v-Ki-ras probe was also found to be enhanced more than 4-fold. NIH/3T3 cells transfected with high molecular weight DNA of these cell lines showed a low frequency of transformed foci, and produced subcutaneous tumors in NIH Swiss nude mice. These transformed cells are being characterized.

Karyotype analysis of these cell lines indicated that they are hypotriploid with possible translocations and various marker chromosomes.

Chromosome 12, in which Ki-ras-2 is located, was found to be trisomic in most cells of Tera-2 but not Tera-1. The latter, however, exhibited insertions in its short and/or long arms of chromosome 12 suggesting possible translocations.

The other human tumor cell lines and murine tumors have not shown remarkable amplification or rearrangement of various oncogenes tested so far, except for a possible amplification of c-fos and enhanced expression of its mRNA in a human choriocarcinoma cell line, and an enhanced expression of c-fos and c-N-ras mRNA in a human hepatoma cell line (J7).

It is of interest to note that a human hepatoma cell line (J2-23) originally passaged in nude mice produced a tumor which upon in vitro culture manifested mouse cell characteristics as evidenced by karyotype analysis and murine ecotropic virus production. This cell line induced only a small tumor when reinoculated into nude mice. This tumor was cultured again and passaged in nude mice to select for a more oncogenic line. When the DNA extracted from this in vivo selected tumor cell line was tested for human alu sequence by hybridization, it was found to be positive.

2. Roles of mos and Other oncogene Expression in the Anchorage-Independent Growth of S+L- Mink Cells Superinfected with a Novel Dual-Tropic Retrovirus. We have isolated two strains of dual-tropic virus from our stocks of B-tropic virus. Unlike most known dual-tropic viruses, these new strains exhibited B-tropism (rather than N- or NB-tropism) and a broad host range similar to xenotropism. Various S+L- cells such as H-MSV-NRK, Ki-MSV-NRK, MoMSV-Mink (S+L- Mink) could be infected with the novel virus and the MSV genome could be rescued. However, it was observed that only the infected S+L- mink cells could give rise to transformed cells which were easily detached spontaneously from the substratum. A clone of such anchorage-independent (AI) cells was isolated and compared with a clone of infected, transformed cells still showing anchorage-dependent (AD) growth. The AI clone grew in suspension in culture medium, showed high oncogenicity in the nude mouse assay, and relatively high efficiency of colony formation in soft agar medium.

By blot-hybridization with v-mos as a probe, the chromosomal DNA of the AI clone, AD clone, and uninfected S+L- mink, as well as ininfected mink lung cells were compared for the intensity and pattern of hybridization . It was observed that, with EcoRI-cleaved DNA, the uninfected mink lung cells showed a single 4.9 kb fragment of c-mos, wheras the uninfected S+L- mink showed an extra 7.3 kb fragment of v-mos, the AD clone showed 2 extra v-mos fragments which were 26 and 7.3 kb each, and the AI clone showed 4 extra v-mos fragments measuring 26, 8.8, 7.3, and 5.6 kb each. Similar trends in the number of v-mos fragments were observed with these DNAs treated with other restriction enzymes such as BamHI and HindIII. It is evident that the v-mos is greatly amplified in the AI clone as compared with AD clone or others.

Furthermore, as would be predicted, the amounts of mRNA expressed by these cells paralleled those of v-mos copies in the DNA, i.e., as compared with the uninfected S+L- mink cells, there was 8-16-fold increase in the amount of RNA produced by AI clone, and 4-fold increase of mRNA expressed by AD clone.

3. Regulation of Viral Replication in Murine Trophoblastic Tumor Cells.

Although murine trophoblast cells are nonpermissive for replication of retrovirus, it can be shown that the virus can be integrated in the cellular DNA and, by treatment with iodo-deoxyuridine or azacytidine, the integrated virus can be activated. Clones of these cells initially infected with Friend leukemia virus (FLV) were isolated, and their high molecular weight DNAs were tested for hybridization with FLV probe. The endogenous viral DNA hybridizing with the FLV probe could be distinguished from the FLV-specific band which showed as a 3.0-3.2 kb fragment after BamHI cleavage. With an eco-specific probe, the FLV-specific band could also be demonstrated as a 10 kb EcoRI fragment. One of the infected clones was fused with a permissive cell line, LMTK-, and several hybrid clones were obtained by selection in HAT medium. These clones were shown to retain the FLV provirus as demonstrated by blot-hybridization of their DNA preparations, and to exhibit no replication of FLV as detected by XC test, reverse transcriptase, and immunofluorescence assays. Therefore, it is not due to the lack of enzyme or some other cellular products in the trophoblast cells that confers them the property of nonpermissiveness, because the deficiency would have been corrected in the hybrid cells. It is possible that there is a cis or trans-acting suppressor in the nonpermissive parent trophoblast cells, which continues to be active in the hybrid cells, and such a suppressor may cause nonfunctioning of viral promotor/enhancer element in LTR in these cells.

A polyoma virus mutant which can replicate in an embryonal carcinoma cell line, F9, was shown in our previous experiments to be able to replicate in trophoblast cell lines, but mutants that can replicate in the latter were unable to do so in F9 cells. In collaboration with Dr. Ito, the promotor/enhancer region, BamHI-PvuII fragment, was ligated 5' to the chloramphenicol acetyl transferase (CAT) gene in a plasmid. F9 cells were successfully transfected with this recombinant DNA (PyF9-90-CAT), and the CAT activity could be demonstrated. However, trophoblast cells similarly transfected did not show CAT activity. In order to examine whether trophoblast cells can take up exogenoud DNA by transfection procedures, a recombinant DNA containing a selectable marker was used. It was found that pSV2-neo DNA can be transfected into trophoblast cells and neomycin-resistant cells could be isolated, albeit with lower efficiency as compared with NIH/3T3 cells. Work is in progress to repeat transfection experiments with PyF9-90-CAT and other constructs of recombinant DNA.

4. Production of Monoclonal Antibody against HTLV Antigens and Screening of Human Antibodies against HTLV. In order to study the antigenic determinants of HTLV-I which are important for viral pathogenesis, transmission, and gene control, attempts have been made to produce monoclonal antibodies against structural as well as nonstructural antigens of HTLV-I. BALB/c mice were repeatedly immunized with washed cell suspensions of MT-2, an HTLV-I-producing human cell line established by immortalization of primary T lymphocytes after cocultivation with an HTLV-I-producer cell line. The hyperimmunized mouse spleen cells were fused with a murine palmacytoma cell line, and cultivated in HAT medium. Hybridoma cell clones producing HTLV-I antibody were screened by ELISA method using lysed, purified HTLV-I particles as well as MT-2 cells as antigens. In addition, a nonproducer lymphoid cell line, C81-66-45, was also used, because this cell line was reported to contain p42 viral protein which is possibly encoded by pX region and may have the ability of trans-acting

transcriptional activation of LTR (Sodroski et al. 1984). Among 650 hybridoma clones that were repeatedly screened for antibody, 9 showed reactivity against lysate of purified HTLV-I, 16 were positive against MT-2 cell antigens, and 52 reacted against C81-66-45 cell antigens. Some of the hybridomas showed reactivity against all these three, and some reacted only with one or two of these three.

A serological survey initiated last year on a sample of Washington, D.C. population was completed. Results showed that 17% (22/130) of the drug addicts possessed antibody reactive against HTLV-I, while 5.3% (6/113) of healthy individuals, 4.3% (8/187) of male homosexuals, and 9.0% (14/155) of chronic disease patients showed positive antibody tests. These positive serum samples will be used in future for HTLV-I antigen analysis together with the monoclonal antibodies.

A serological survey using AIDS-virus antigen and ELISA method was also conducted on the male homosexuals. The antibody positive rate was 4.3% (8/187) in male homosexuals, and 0.9% (1/113) in apparently healthy individuals. The titer ranged from 10-1600 for the homosexuals and was 25 for the normal individual.

5. Ability of Various Nonlymphoid Cell Lines to Support Replication of HTLV-I. Various human and animal nonlymphoid cell lines such as XC (rat, RSV-transformed), 81 (cat, MSV-transformed), KC (human, RSV-transformed), D17 (dog osteosarcoma) and DoCl.S+L- (dog, MSV-transformed) cells were infected by cocultivation with Cs-137-irradiated MT-2 cells, or infected with a concentrated HTLV-I. These cells were maintained for several months, and although most of them have not shown signs of HTLV replication, some clones of XC and D17 cells continued to show syncytial cells. Their high molecular weight DNA as well as mRNA were tested for hybridization with a P-32-labeled HTLV-I probe, pCH, which encompasses env and a portion of pol genes. Although neither the high molecular weight DNA nor the mRNA showed any significant amount of hybridization with pCH probe, the presence of defective or mutant HTLV-I which could not be detected by this probe cannot be excluded.

Significance for Biomedical Research and the Program of the National Cancer Institute:

An activated c-KI-ras-2 gene has been found in various human tumor cell lines derived from lung, colon, pancreas, gall bladder and ovarian carcinomas, rhabdomyosarcoma, and acute lymphocytic leukemia, but so far none from teratocarcinoma has been reported. The findings that NIH/3T3 cells transfected with DNA of teratocarcinoma cell lines showed morphological transformation and produced tumors in nude mice suggest the possibility that c-Ki-ras-2 gene is activated in these lines. In addition, amplification and rearrangement of this gene are demonstrated on both of the two cell lines tested, although they differ in cell morphology and karyotype. Chomosome 12 in which c-Ki-ras-2 gene is located was found to be trisomic in most cells of Tera-2 but not in Tera-1, the latter exhibiting insertions and homogeneously staining regions in chromosome 12. The mechanisms leading to activation of c-Ki-ras-2 may be different for those responsible for amplification, and may also be different among different teratocarcinomas. Thus, effects of oncogene amplification may not

represent a single gene dosage phenomenon: mutation within the amplified genes, translocations to new chromosomal contexts, or insertions within the target chromosome, or coamplification of adjacent genes may have additional influences.

Although it is not known whether these teratocarcinoma cells can be induced to differentiate into nontumor cells as some pluripotent murine teratocarcinoma cells do, it would be most intriguing if the activated oncogene would revert to inactive form after the tumor cells were induced to differentiate into normal cells. The important, old question whether epigenetic changes play any role in oncogenesis may find some clue in these lines of investigation.

Although the mechanism for differentiation of cells is not fully understood in molecular terms, it is possible that chromosomal recombination, gene rearrangement, or changes in methylation status of nucleotides, which are associated with differentiation processes, may result in inactivation of an active oncogene by frame shift, by generation of suppressor substance, or by removal of enhancer elements. Activation of a proto-oncogene may also entail switching off of another oncogene which has been active.

Elucidation of the mechanisms by which S+L- mink cells become anchorage-independent (AI) in their growth pattern after superinfection with a new dual-tropic virus would contribute in understanding the function of mos and other oncogenes and growth factors which would be responsible for initiating and maintaining AI status of growth. Since high oncogenicity was associated with the AI growth pattern, the possibility of association between metastasis and excessive oncogene expression may be considered.

Availability of polyoma virus mutants that have enhancer elements functional in certain types of cells, such as embryonal carcinoma and trophoblast cells, may provide a system to test the tissue specificity of enhancer functions in the LTR and the requirements for leukemogenic potential of retroviruses. Analysis of methylation status and transcriptional activities of retroviral provirus in the permissive, or nonpermissive, parent cells and their hybrids derived by cell fusion, may facilitate understanding of the mechanisms of regulation of gene replication and expression in these systems.

Although the HTLV-I gag gene products p19, p24 and p15, and pol gene product have been well characterized, the identification and purification of env and pX gene products have been difficult. Monoclonal antibodies against p19 and p24 have been reported, but those against other gene products remain to be developed. Such reagents would be useful in: 1) antigenic analyses of HTLV structural and precursor viral antigens; 2) increasing the capabilities for detecting and diagnosing HTLV infection; 3) elucidating the biological functions of env and pX gene products, such as infectivity, transformation, immunosuppressive functions, and transcriptional activation, etc.; 4) distinguishing subtypes of HTLV; and 5) therapeutic and preventive applications.

Studies on the in vitro transmission of HTLV-I to nonlymphoid cells of human and animal origin would help clarify the mechanisms of tropism, permissiveness, and regulation of HTLV replication, and, if viral mutants were

isolated, they would contribute greatly to the understanding of various activities of HTLV.

Proposed Course of Project:

1. Tumor cells from nude mice inoculated with the NIH/3T3 cells transfected with Tera-1 or Tera-2 DNA and the in vitro transformed NIH/3T3 cells will be tested again for human alu sequence, and if confirmed to be positive, a second round of DNA-transfection experiment will be performed. Further identification of the activated oncogene will be done by hybridization with various v-onc probes. The activated oncogene, which may or may not be Ki-ras-2, will be isolated by cloning and its nucleotide sequence determined. Since Ki-ras-2 gene is amplified, the amount of p21 protein encoded by this gene will be quantitated by immunoprecipitation-autoradiography or Western blot-ELISA test. If possible, tissues of teratocarcinoma may be obtained from patients and their DNA and mRNA tested as above. Patients' peripheral lymphocytes or normal tissue may serve as a control. Portions of these cells will be cultivated, and maintained in vitro to follow subsequent changes. Further work similar to that mentioned above, is also needed to characterize and isolate the putative human hepatoma oncogene which may have inadvertently been integrated into the cells of nude mouse in vivo conferring upon them the neoplastic property.

2. The findings that mos was amplified and excessively expressed may not necessearily account for the anchorage-independent (AI) growth of the S+L- mink cells superinfected with the new dual-tropic virus. Other oncogenes that may be involved separately or in cooperation with mos should be considered. Study of the role of mos-encoded protein in the phenotype of AI growth in relation to cytoskeletal proteins and growth factors will be performed. These results may throw light on the physiological functions of mos in normal cells. The question whether the unique dual-topic viral genome or its products, or the cell receptor for this virus, may have a role other than amplification of mos in the induction of AI growth capability will be investigated.

3. The enhancer region of mutant polyoma viruses that can grow in trophoblast cells but not in F9 cells will be compared with the enhancer region of those mutants that can grow in F9 as well as trophoblast cells for their ability to enhance expression of CAT gene which is ligated to the 3' end of enhancer element derived from these mutants. It is also planned to modify LTR region of retroviruses by deletion followed by substitution with enhancer element of polyoma virus mutants. Tissue-specificity and leukemogenicity of these modified retroviruses will be tested.

Clones of hybrid cells produced by fusion of FLV-provirus-carrying trophoblast cells with LMTK cells will be studied further for their FLV-inducibility, and compared with their parent trophoblast cells for production of transcripts and proteins encoded by FLV.

4. By using the monoclonal antibodies produced by hybridomas screened so far, efforts will be directed to identifying and characterizing the HTLV antigens by means of Western blot-ELISA, immunofluorescence and viral neutralization tests. Human serum samples reactive against HTLV-I or AIDS-virus will be tested in parallel for characterization of antigens.

5. Another HTLV-I probe, pMT-2, which includes the entire HTLV-I genome, will be used for hybridization tests to detect any defective or mutant HTLV genome present in these nonlymphoid cells. The proteins produced by the putative defective or mutant virus may provide a tool for dissecting the viral antigenic structure when combined with the use of monoclonal antibodies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04834-09 LCO

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Genetic mechanism of carcinogenesis and biological modifiers as defense mechanism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S. S. Yang, Research Chemist, LCO, NCI

Other: J. V. Taub, Biolab Technician, LCO, NCI
R. Modali, Biologist, LCO, NCI

COOPERATING UNITS *(if any)*

C. C. Ting, Immunology Branch, NCI
G. C. Yang, OBCB, DCH, CFSAN, FDA, DHHS
P. Yasei, OBCB, DCH, CFSAN, FDA, DHHS

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20205

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2.5	1.0	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The thrust of this study is to elucidate the genetic mechanisms of neoplastic transformation of normal tissues and the cellular immune defense mechanisms against neoplastic cells. Two experimental systems were used: 1) A human oncogene derived from a hepatocellular carcinoma currently established in tissue culture; 2) T-cell growth factor, an autocrine of T-cells, and CCDF (cytotoxic cell differentiation factor), produced by macrophages for the induction of natural killer cells to become cytotoxic cells.

A systematic screening with DNA transfection assay, preferential AFB-1 binding and repeated molecular clonings were carried out and led to the successful isolation of a human oncogene, PM-1, capable of cell transformation and its cell transformation capability was enhanced by more than 300 fold upon AFB-1 activation. PM-1 was 3.1 kb and shared some homology with the ras oncogene. We have done a restriction map of PM-1 and have also resolved the nucleotide sequence of approximately 65% of the entire gene; the latter verified the accuracy of the restriction sites along the genome. We also pinpointed some of the deoxyguanine targets in the PM-1 nucleotide sequence under controlled binding conditions with AFB-1 epoxide, yielding some relevant information regarding nucleotide sequence specificity influencing carcinogen activation of an oncogene.

After extensive purification, the function and relationship of two oncogene products, IL-2 and CCDF, after extensive purification have been resolved. In the absence of antigenic or mitogenic stimulation, IL-2 provides the first signal to activate the cytotoxic precursors and prepares these cells to differentiate into cytotoxic effectors in the absolute presence of CCDF. The sequential actions of these two lymphokines are critical for the generation of lymphokine-induced cytotoxicity, a cellular process considered pivotal in cellular immune defense mechanisms against tumor cells.

GPO 914-918

Project Description

Objectives:

1) To resolve the molecular identity and the nucleotide sequence of a human oncogene, PM-1.
2) To study the chemical activation of AFB-1-epoxide with the deoxyguanine residues along the PM-1 DNA and the activation of PM-1 oncogene.
3) To purify and to elucidate the respective function of IL-2 and CCDF as biological modifiers in cellular immune defence mechanism.

Methods Employed:

Multidisciplinary approaches involving nucleic acid and protein chemistry, recombinant DNA, and tissue culture were employed.

Major Findings:

1. PM-1 human oncogene restriction map, nucleotide sequence and homology to other oncogenes. Human transforming DNA sequences related to the known oncogene families have been detected by others in various human tumors obtained from biopsy or tissue culture cell lines. We have isolated a human oncogene from a hepatocellular carcinoma obtained from an African patient (Mahlavu); the tumor had recently been established in tissue culture, MAH, and had no hepatitis B marker on the cell surface. Our earlier finding established the capability of high molecular weight (HMW) hepatocellular carcinoma DNA to mediate cell transformation in NIH/3T3 cells at a low efficiency. However, upon binding with AFB-1-epoxide MAH HMW DNA showed greatly increased cell transformation efficiency (10-20 fold). AFB-1 bound DNAs from NIH/3T3 DNA, E. coli, normal human liver DNA, Jane Alexander hepatoma cell line and c-K-ras DNA all failed to show cell transformation inspite of successful AFB-1 binding. A systematic approach using AFB-1-epoxide binding on subgenomic MAH DNA fragments for effective screening and transfection assay on NIH/3T3 cells, was carried out to identify the MAH transforming DNA. With repeated clonings and screenings a 3.1 kilobasepair (kb) Hind III genomic DNA fragment, PM-1, was successfully cloned at the Hind III site of pBR 322. PM-1 showed approximately 10 fold greater efficiency in transfection assay. Upon controlled activation with AFB-1-epoxide, PM-1 DNA showed more than 300 fold increase in cell transformation capability (Publication 1).

Detailed homology analyses were carried to identify PM-1 DNA with members of known oncogene family such as Harvey ras (H1), Kirsten ras (KBE-2; HiHi-3), c-Kirsten-ras-1, and n-ras. Under stringent hybridization condition, only K-ras (KBE-2), H-ras and n-ras hybridized with PM-1 DNA. Essentially extremely poor or no hybridization was observed with the cK-ras-1 and HiHi-3, a 1.0 kb subgenomic fragment of K-ras. We then attempted to define the homology areas by S-1 nuclease protection analysis. Results indicated that basically the homologous sequence resides withing the Pst I-Hind III 2.05 kb fragment of PM-1 DNA. Approximately 65% of the entire PM-1 DNA has been sequenced. Nucleotide sequence within the Pst I-Hind III fragment showed homology with H-ras, K-ras and n-ras interrupted with insertions of divergent sequences and inversions.

A restriction endonuclease map of PM-1 has been constructed for 12 enzymes: Ava I, Ava II, Bgl I, Eco R I, Hpa II, Hind III, Mnl I, Nar I, Pst I, Pvu II, Sst I, and Xba I. The nucleotide sequence of PM-1 that has been resolved thus far confirmed the locations of the deduced restriction sites.

Our nucleotide sequence data showed the existence of a human viral LTR homologous to the novel human endogenous retroviral LTR reported recently (Sci. 226:1204-1206, Dec. 1984) for ERV3 from a human recombinant DNA library using probes from two regions of the type C baboon endogenous virus (BaEV) genome. Some divergence and deletion of certain sequences do exist. PM-1 LTR showed at least 2 copies of TATAA and CCAAA sequences, the transcriptional signals, thus defining a probable U3 region.

Currently we are attempting to use deletion mapping to further localize the transforming sequence of PM-1 within the 3.1 kb fragment.

2. Preferential binding of AFB-1-epoxide with dG residues along the PM-1 DNA and activation of the oncogene. It has been proposed that AFB-1 showed preferential binding to nucleotide sequences that showed alkali sensitivity. We reported preferential binding of AFB-1-G into certain subgenomic DNA fragments. AFB-1-epoxide tended to bind stoichiometrically with the deoxyguanine residue of any DNA tested (publication 1) until most or all dG residues become bound. However at lower concentrations, i.e. low AFB-1 femtomole/ug DNA ratio, which corresponds to concentrations observed in vivo, we found that AFB-1-epoxide tended to bind in the following fashions: 1) most preferred dG when it is flanked by dC and dG; 2) moderately preferred when dG is preceded by dA or dG and followed by dC and 3) the least preferred if dG is flanked by dA or dT and dT or dA. We have localized some of the dG targets within the PM-1 DNA preferentially bound by AFB-1-epoxide under limiting concentration of AFB-1. We found loci of dG residues that led to "over-kill" of all biological activity when attacked by AFB-1-epoxide. On the other hand, we have not yet been able to correlate the significance of oncogene activation to any of the preferred dG target of AFB-1-epoxide under limiting concentration of AFB-1 (Publication 2).

3. Purification and elucidation of the respective function of IL-2 and CCDF as biological modifiers in cellular immune defense mechanism. In a continuing collaboration with Dr. George C. Ting of the Immunology Branch, we have purified the murine Il-2 and CCDF (the latter is a cytotoxic cell differentiation factor) by sequential amonium sulfate precipitation, sephadex G200 gel filtration, DEAE cellulose column chromatography for Il-2 but sodium silicate powder absorption for CCDF followed by two successive HPLC (C10 and Diphenyl) column chromatography using gradient elution with acetonitrile. IL-2 was purified approximately 75 fold and CCDF was purified at least 2000 fold. The purified protein bands that constituted full activity of CCDF resided in two molecular species of 23 K dalton and 45 K daltons when assayed in a reconstituted condition. This suggests that there may exist a family of CCDF molecules as observed murine in IL-2.

We reported last year the function of IL-2 in the activation of T-cell differentiation into antigen-specific cytotoxic T-cell (CTL) and the subsequent expansion of the antigen-specific CTL population. During the process we

observed that cytotoxic cells could be induced against various tumor cell targets by culturing normal spleen cells with syngeneic peritoneal macrophages and indomethacin. Antigenic or mitogenic stimulation is not essential in this induction of what we currently term lymphokine-induced cytotoxic cells (LICC), which may serve as an important cellular immune defense mechanism against spontaneous neoplasm. We eventually isolated and purified a lymphokine(s), CCDF, produced by peritoneal, unstimulated macrophage in the mouse, that is reponsible for the generation of LICC. During the induction of LICC in normal spleen cells, CCDF functions in a synergistic manner with IL-2, the latter being at suboptimal concentration and thus effectively eliminating the involvement of T-cell. That IL-2 at a suboptimal level, is an obligatory requirement for the induction of LICC was established by virtue that anti-IL-2 completely blocked the induction of LICC. The effectors of LICC were found to be Thy+, Lyt2- and AGM1- and thus presented themselves as a unique class of cytotoxic cells of neither classical CTL nor NK cells. The precursors were, however, AGM1+ and Lyt2-, which were consistent of being NK-like cells. Detailed analysis of the LICC induction process revealed a critical temporal relation between the actions of IL-2 and CCDF. An initial transient activation phase lasting 4 hrs., in which IL-2 provided the first signal to prepare the precursors to enter into the CCDF-dependent differentiation phase in which CCDF provided the second signal to induce the IL-2-activated precursors into cytotoxic effectors, with this process requiring 48 hrs. to complete. The sequential presence of these lymphokines at an appropiate time during the activation and differentiation phases is critical for the generation of LICC response (Publications 3 and 4).

Significance to Biomedical Research and the Program of the Institute:

PM-1, the human oncogene isolated from a hepatocellular carcinoma cell line, may ultimately turn out to be a novel oncogene since it shares only limited homology with the ras family. Moreover the fact that it can be activated by AFB-1-epoxide to become a potent transforming gene may represent a significant contribution to our understanding of the mechanism by which a chemical is oncogenic.

The lack of immunogenicity in spontaneous neoplasm and the fact the tumor cell frequently induces immunosuppression in the tumor-bearing host constitute the major dilemma confronted in immunotherapy of cancer. The mode of action and the induction of LICC may serve to resolve some of these problems by virtue that LICC selectively kill lymphoid or solid tumor target cells. A preliminary animal trial also showed that LICC also possess in vivo anti-tumor activity. LICC may ultimately lend itself as an effective adjuvant therapy of tumor growth.

Proposed Course of Research:

The work is in progress.

Publications:

Yang, S.S., Taub, J.V., Modali, R., Viriera, W., Yasei, P. and Yang, G.C.: Dose-dependency of aflatoxin B-1 binding on human high molecular weight DNA in the activation of a proto-oncogene. Environmental Health Perspectives, Vol. 62, ed. F. de Serres and W. Sheridan. July, 1985.

Modali, R. and Yang, S.S.: Specificity of aflatoxin B-1 binding to human proto-oncogene nucleotide sequence. In: Symposium on Monitoring of Occupational Genotoxicants, ed. by H. Norppa and M. Sorsa. A. R. Liss, Inc. N.Y., N.Y. (Submitted)

Yang, S.S., Malek, T.R., Hargrove, M.E. and Ting. C.C.: Lymphokine-induced cytotoxicity: requirement of two lymphokines for the induction of optimal cytotoxic responses. J. Immunology. 134:3912-3919, 1985.

Ting, C.C., Yang, S.S. and Hargrove, M.E.: Lymphine-induced cytotoxicity: Characterizations of effectors, precursors and regulatory anxillary cells. European J. Immunology. (Submitted)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04833-16 LCO

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biological studies of various normal, virus-infected, and malignant cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: N. A. Wivel, Senior Investigator, Laboratory of Cellular Oncology, NCI

COOPERATING UNITS *(if any)*
P. M. Pitha, Associate Professor, Departments of Microbiology and Oncology, Johns Hopkins University School of Medicine, Baltimore, MD

LAB/BRANCH
Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.00	1.00	0.00

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It is the primary purpose of this project to study some of the pertinent factors which influence cell differentiation and malignant transformation, using techniques and approaches which range from the microscopic to the molecular level. Particular emphasis is given to those systems in which murine RNA tumor viruses or chemical carcinogens may be the transforming agent. A variety of mouse model systems are used, including methylcholanthrene-induced sarcomas, plasma cell tumors, mammary tumors, and neuroblastomas. Current projects include; 1) effects of interferon on methylcholanthrene-induced sarcomas of the BALB/c mouse with the aim of defining antitumor activity and relationship to immune response; and 2) effects of interferon treatment on NIH 3T3 cells transfected with various (Ha) ras related oncogenes.

Our results suggest that the major effects of interferon on chemically-induced sarcomas do not appear to be mediated through anticellular activity, but are related to the immune response in the host animal. A number of experiments confirm the necessity of functional T cells in order for interferon to exert its antitumor effect. Since interferon increases the expression of H-2 antigens on these tumor cells, this may serve to enhance their recognition by host T cells.

NIH 3T3 cells were transfected with either cellular or viral Ha-ras genes prior to interferon treatment. In most of the cell lines examined, interferon inhibited cell growth, reduced cloning efficiency and delayed, but did not prevent, tumors in nude mice. When in vitro oncogene expression was measured at the mRNA level, c-Ha-ras mRNA was reduced by 2 to 4 fold following interferon treatment, while the mRNA for H-2 antigen was increased by 3-4 fold.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

All of these projects have the fundamental aim of more precisely defining the mechanisms by which interferon acts on cells.

Methods employed:

The current projects require a variety of *in vitro* and *in vivo* techniques including cell culture, cytotoxicity assays and growth assays of methylcholanthrene-induced sarcomas in both BALB/c mice and nude mice. Expression of H-2 antigens is measured by radioimmunoassy and analysis of RNA using a ^{32}p labeled H-2 probe in a dot blot assay. The presence of tumor-specific transplantation antigens is measured by immunizing mice with irradiated tumor cells, followed by subsequent challenge with live tumor cells.

Major Findings:

1. Effects of interferon on methylcholanthrene-induced sarcomas of BALB/c mice. The observed effects of interferon on cells are the result of a sequence of events such as interaction with a membrane receptor, establishment of an antiviral state, inhibition of cell growth or modulation of differentiated functions. Interferon induced effects can be divided into four different categories: 1) membrane alterations; 2) changes in the cytoskeleton; 3) induction of new enzymatic systems; and 4) inhibition of inducible proteins. While the induction of new enzymatic proteins seems to play a major role in the antiviral effect of interferon, the relevance to the antitumor effect of interferon is not clear.

The rationale for pursuing these studies was predicated on a number of distinct but related factors. First, the clinical literature regarding the interferon treatment of various human sarcomas is less than definitive. Thus one could expect to derive a more interpretable set of data regarding properties of interferon in a controlled animal model system maintained in syngeneic mice. The methycholanthrene-induced sarcoma, Meth A meets the aformentioned requirements; it can be passed both *in vivo* and *in vitro* and passage *in vitro* does not attenuate the tumorigenicity of these cells. There are no known murine retroviruses or other infectious viruses associated with this tumor and thus one can easily dissect the anticellular effect of interferon from the antiviral one. By comparing the response in cell culture with that in the intact mouse it is possible to analyze the direct action of interferon on Meth A cells separately from the host mediated effects. It is of importance that there are stable membrane markers such as the tumor specific transplantation antigen (TSTA) and the H-2 antigens which are potentially susceptible to the action of interferon.

Our initial series of experiments established that *in vitro* treatment with a mixture of alpha and beta interferon caused cytostasis but was not cytotoxic, while gamma interferon was more efficient than the alpha-beta mixture. Cell growth was fully restored following removal of interferon from the system. When treated cells were used to challenge mice, the development of tumors could be prevented as long as the challenge dose was 10^5 cells or less. The interferon

effect could be abrogated by using a dose of 10^6 cells. Meth A cells treated with alpha-beta interferon were rejected more efficiently than those treated with gamma interferon. These results suggest that the major effects of interferon on Meth A cells may not be mediated through anticellular activity, and thus a number of experiments were done to define the role of the immune response in this system.

Challenge of nude mice with virus-infected tumor cells serves as a stimulus for natural killer (NK) cell activity and leads to tumor rejection. We elected to compare the tumorigenicity of interferon treated and untreated Meth A cells in nude mice with that seen in syngeneic BALB/c mice to determine whether interferon treatment, in the absence of virus, could serve as a stimulus for NK cell activity. After a challenge dose of 10^5 cells, both control and experimental groups had a tumor incidence of 100%. With a challenge dose of 10^4 cells, 100% of the controls and 85% of the mice receiving interferon-treated cells developed tumors. As opposed to BALB/c mice, interferon offered no protective effect in nude mice, indicating that the rejection of interferon treated Meth A cells is thymus dependent, and do not mediated by the NK system.

The dependence of the protective effect of interferon on the presence of functional T cells was further tested in mice in two ways. First, mice were immunosuppressed with cyclophosphamide at a dose just below the lethal level, but sufficient to produce long-term suppression affecting all classes of anti-body-producing cells. Under these conditions the results paralleled those seen in nude mice, i.e., the interferon effect was abolished. Second, mice were immunosuppressed with cyclosporin A, an agent which has been shown to preferentially affect T cells. Again the effect of interferon was negated.

Since many tumors in mice carry retroviruses or express retroviral antigens, we attempted to answer the question of whether infection with ecotropic virus (Moloney MuLV) could enhance or otherwise alter tumor cell recognition and whether it could amplify the interferon effect. Meth A cells were infected in vitro and after several passages it was established that 95-100% of the cells were producing virus. When transplanted into syngeneic mice such infected cells were rejected even without prior interferon treatment. This host response to infected cells was probably related to the antigens associated with Moloney MuLV. Friend, Moloney, Rauscher and Rich viruses are all antigenic in the mouse and vaccination has resulted in protection against challenge with active virus. Conversely, Gross MuLV does not elicit such an antibody response. Accordingly, Meth A cells were infected with a B-tropic variant of Gross MuLV, 1802-B, which is infectious in BALB/c mouse cells. When Meth-A cells infected with this virus were used to challenge mice, the results were the same as with uninfected controls.

Our recent results indicate that interferon treatment of Meth A cells increases the expression of H-2 antigens. Since the products of the major histocompatibility complex have a critical role in the initiation and regulation of the immune response, it is possible that at least some of the effects of interferon on these particular tumor cells may be due to alterations in the H-2 complex. Results from other laboratories suggest that H-2 antigens play a role in the host recognition of the TSTA of methylcholanthrene-induced sarcomas. It has to be considered that increased H-2 expression could facilitate the

recognition of Meth A cells by an appropiate subpopulation of T cells.

Current efforts involve the use of an in vitro cytoxicity assay to aid in the identification of splenic T cells which have the capacity to lyse Meth A cells. Initial efforts failed to demonstrate effector T cells, but present experiments include the pretreatment of Meth A cells with such agents as concanavalin A and neuraminidase in order to make them more susceptible to lysis. There is some preliminary evidence that peritoneal exudate cells, when properly stimulated may be more effective than spleen cells in attacking methylcholanthrene-induced sarcomas, and these findings are being repeated in our laboratory.

2. Effect of interferon treatment on NIH 3T3 cells transfected with ras oncogenes. Under certain conditions interferon can be shown to cause a reversion of NIH 3T3 cells which have been transformed by oncogenes, and there are data which suggest that interferon can prevent stable integration and expression of transfected plasmids containing thymidine kinase or dihydrofolate reductase genes. In order to further characterize these putative properties of interferon, we have analyzed several lines of NIH 3T3 cells which have been transfected with ras-related oncogenes.

The cell lines which were examined had been transformed with the v-Ha-ras gene or with the mutated proto-Ha-ras gene from the human bladder carcinoma cell line, T-24; the latter gene was under the control of an SV40 promotor. In essentially all of the cell lines examined, in vitro interferon treatment inhibited cell growth, reduced the cloning efficiency and decreased colony formation in soft agar. When treated cells were transplanted into nude mice, there was a delay in the appearance of tumors as compared to the controls, but there was no reduction in incidence.

Alteration of oncogene expression was determined by hybridizing ^{32}p-labeled DNA probes of several oncogenes to the RNA of the various transformed NIH 3T3 cell lines. The following oncogenes were tested: C-Ha-ras, v-Ha-ras, c-myc, c-myb, v-fos, and p^{53}. RNA was assayed following 48 hours of treatment with alpha-beta interferon and again after two weeks of treatment. The levels of c-Ha-ras and c-myc RNA were reduced 2 to 4 fold following long term treatment with interferon while H-2 RNA levels increased. There was no effect on any of the other oncogenes.

Although our experiments did not detect any reversion of previously transformed cells, the timing of interferon treatment was significant. If NIH 3T3 cells were pretreated with interferon prior to transfection with v-Ha-ras DNA and then treated post transfection for 92 hours, there was a 6 fold reduction in the number of transformed foci. If interferon treatment was continued for 2 weeks, transformed foci were reduced 15 fold. This reduction could not be explained by simple inhibition of cell growth, and thus interferon may have a role in inhibiting the phenotypic expression of transfected oncogenes. Experiments in progress are directed toward defining some of the mechanisms that account for these results.

Significance to Biomedical Research and the Program of the Institute:

A considerable amount of recent clinical research has been devoted to studying the antitumor effects of interferon. Whether this family of compounds will be particularly useful, remains to be determined. However, our work done with mouse model systems has suggested some parameters which are important in the use of these agents. In particular, immune competence is required if interferon is to inhibit growth of some neoplasms.

Proposed Course of Research:

The work is in progress.

Publications:

Wivel, N. A., Glasgow, G., Vengris, V. E., and Pitha, P. M.: Relationship of T-cells and retroviral infection to the antiproliferative properties of mouse interferons. Int. J. of Cancer; (in press).

SUMMARY REPORT
LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE
October 1, 1984 to September 30, 1985

The Laboratory of Pathology is responsible for all the diagnostic services in anatomic pathology for the Clinical Center of the NIH and has research programs in various areas of experimental pathology. A fully accredited residency program in anatomic pathology is provided for 9 residents. The Laboratory is divided into 6 sections:

A. Surgical Pathology and Postmortem Section (Dr. Ernest E. Lack, Chief)
B. Cytopathology Section (Dr. Elizabeth W. Chu, Chief)
C. Ultrastructural Pathology Section (Dr. Timothy J. Triche, Chief)
D. Biochemical Pathology Section (Dr. David A. Zopf, Chief)
E. Tumor Invasion and Metastases Section (Dr. Lance A. Liotta, Chief)
F. Hematopathology Section (Dr. Elaine S. Jaffe, Chief)

All sections conduct investigative work and provide research opportunities for the residents. Investigative work completed or in progress is listed by section as follows.

A. Surgical Pathology and Postmortem Section

5,500 surgical specimens or biopsies (53,000 slides) were accessioned in the past year. Approximately 1,000 specimens of fresh human tissues, including the eyes which are regularly removed during a complete autopsy, were furnished to NIH scientists in various laboratories. A tissue procurement nurse works closely with the staff and helps in the distribution of specimens to scientists. Clinicopathological studies in pediatric neoplasms, endocrine tumors, soft tissue sarcomas, acquired immune deficiency syndromes and breast cancer are in progress. Dr. Lack, chief of the section, has published a series of clinicopathologic case studies in germ cell tumors, renal cell carcinomas, and carotid body hyperplasia in cystic fibrosis and cyanotic heart disease.

B. Cytopathology Section

This section provides diagnostic services in cytology (both exfoliative and fine needle aspiration) and medical genetics (service and research). The chromosomal analysis includes conventional and special banding techniques (C-, G-, and R-bands) for the examination of individual chromosome pairs. During the year, approximately 5,000 cytology specimens were accessioned.

In addition to the diagnostic reports, the staff of this section collaborates closely with the clinical and surgical staff in various clinicopathologic research projects. Dr. Sue Ellen Martin has been applying immunoperoxidase techniques to cytological materials. The cytologic diagnosis of malignant lymphoma can be extremely difficult because malignant lymphoid cells can be morphologically similar to reactive lymphoid cells. Dr. Martin has developed a new immunoperoxidase method employing a battery of antibodies to T and B cell markers to distinguish malignant lymphoma cells from reactive cells. Dr. Chu continues the development of the fine needle aspiration technique. Cytogenetic studies are being conducted on an experimental model of progression from non-

metastatic to metastatic tumors. Dr. Chu also serves on the staff of Tutorial on Clinical Cytology, sponsored by IAC, ASC, and AARM. She gives lectures and workshops on fine needle aspiration cytology.

C. Ultrastructural Pathology Section

This section provides diagnostic electron microscopy services for a diverse group of Clinical Center physicians, including NCI, NIAMDD, NHLBI, NIAID, and NINCDS, as well as submitted cases from outside physicians. This past year approximately 250 cases were accessioned; over 200 were processed and diagnosed. This facility provides diagnostic training and clinical research opportunities for residents and fellows. Dr. Maria Tsokos has been doing diagnostic EM while investigating the production of matrix proteins and tissue specific proteins by neuroblastoma cells treated with differentiating agents. The results indicate that neuroblastoma shows tripartite differentiation in vitro into 3 main cell types: neuronal, Schwannian and melanocytic.

Dr. Triche, in collaboration with several other investigators at NIH and at Navy Hospital and at Fordham University, has demonstrated that a unique, previously uncharacterized tumor of childhood, peripheral neuroepithelioma (or peripheral neuroblastoma) is distinct from classic childhood neuroblastoma in all ways. Specifically, it lacks N-myc expression, unlike the majority of neuroblastomas, and instead routinely demonstrates a reciprocal (11:22) chromosomal translocation. C-myc is expressed routinely by this tumor, unlike neuroblastoma. It never produces catecholamines (unlike 95% of childhood neuroblastomas) but instead contains purely cholinergic neurotransmitter enzymes (choline acetyl transferase). No other oncogene has so far been identified. Clinical studies based on these studies are underway, and early results (11 months median follow-up) indicate a prognosis inferior to Ewing's sarcoma (with which it is often confused) and vastly superior to disseminated neuroblastoma (which was the historical diagnosis).

Another area of active investigation by Dr. Tsokos and Dr. Triche is childhood rhabdomyosarcoma. Tumor material has been characterized by immunocytochemistry, tissue culture, and monoclonal antibody studies, as well as conventional histology. Based on information from these studies, two basic types of rhabdomyosarcoma have been identified. A retrospective analysis of 159 cases from NCI and St. Jude Children's Research Hospital has shown strong correlation between these criteria and prognosis, unlike any other criteria previously identified. As a result, an international review panel has been convened by Dr. Triche, Dr. Hal Maurer (Chairman of the Intergroup Rhabdomyosarcoma Study, or IRS), Dr. Giulio D'Angio (President of SIOP, the International Society of Pediatric Oncology), and Dr. William Newton (Pathologist-in-chief in the IRS). This panel will propose morphologic criteria for categorization of childhood rhabdomyosarcoma into favorable and unfavorable histology, substantiated by statistical analysis, and to be employed by all cooperative studies in the western world. This will be the first time such uniformity of criteria for treatment purposes will have been achieved in pediatric oncology.

Dr. Triche's group has determined that the type of matrix molecules produced by pediatric "round cell" tumors can be a clue to their cell of origin. He has characterized a previously undescribed high molecular weight matrix protein. The function and composition of this protein is unknown. Dr. Tsokos has found that neurone-specific enolase is a reliable marker for primitive childhood tumors of neural origin. This marker should be of clinical application by the pathologist in the differential diagnosis of neuroblastoma versus other solid tumors of childhood.

D. Biochemical Pathology Section

The Biochemical Pathology Section is carrying on research on immunochemistry of complex carbohydrates. Current approaches include 1) determination of carbohydrate structures of glycoproteins and analysis of oligosaccharide mixtures by gas chromatography/mass spectrometry (GC/MS), 2) development of hybridoma antibodies against oligosaccharide haptens, and 3) immunochemical studies of cell surface glycoproteins and 4) nucleotide sequencing of hybridoma antibodies of Vh-GAC family.

Carbohydrate chains released by trifluoroacetolysis of whole tissues, tissue fractions, or cells grown in culture, are easily recovered in nearly quantitative yield and reconstituted to their native form. Analysis of the majority of oligosaccharides containing six or fewer monosaccharide units is performed by combined gas chromatography and mass spectrometry of permethylated, N-trifluoroacetylated oligosaccharide derivatives. Analysis for certain specific oligosaccharides is carried out by radioimmunoassay using antibodies produced against purified oligosaccharides coupled to polypeptide carriers. It is anticipated that the repertoire of oligosaccharide chains produced by cells or tissues will reflect states of cellular differentiation and reveal potential cell surface markers.

Membrane glycoproteins behave as either carbohydrate or peptide antigens and occasionally express antigens that combine specific structural elements from both sugar and peptide moieties. Immune responsiveness to cell surface glycoproteins has not been studied systematically. They are characterizing the fine specificities of autoantibodies against glycoproteins of human erythrocytes from patients with altered immunologic states. In addition, they are preparing hybridomas that secrete monoclonal antibodies against various portions of the carbohydrate and peptide moieties of human glycophorin A, the major sialoglycoprotein of human erythrocytes.

The mouse immune response to streptococcal group A carbohydrate (GAC) utilizes many different gene segments from a diverse array of precursor Vh genes. The gene family number is estimated at 10 and the diversity of response in normal mice at over 200 different antibodies. The response is clonally restricted in each mouse immunized, but each mouse appears to express a different clonally homogeneous antibody in its serum in spite of genetically common background. The common denominator of similarity in these antibodies is their usage of highly homologous Vh gene segments, and identical Dh, and Jh segments. The serum response to GAC is entirely deficient in CBA/N mice which makes this an ideal system in which to investigate possible constraints on gene segment utilization.

Laboratory of Pathology, DCBD, NCI

E. Tumor Invasion and Metastases Section

The Section of Tumor Invasion and Metastases is studying the biochemical and molecular genetic mechanisms which play a role in tumor cell invasion and metastases formation. This group has identified three biochemical factors: a) specific new types of proteases, b) a new type of matrix receptor and c) a new type of autocrine motility factor (AMF). All factors are quantitatively enhanced in actively invading tumor cells. Antibodies against the protease react with actively invading breast carcinoma cells in tissue sections, and are therefore of diagnostic usefulness. Blocking the proteases inhibits tumor cell invasion in vitro. Blocking the matrix receptor abolishes experimental metastases in mice. This receptor can also be readily measured in human breast carcinoma tissue. Attempts to correlate receptor content with clinical stage of disease have demonstrated a higher content of exposed laminin receptors in breast cancer cases with 2 or more lymph nodes positive for metastases compared with cases with no metastases. Clinical trials are now being set up to study the localization of metastases by labeled laminin fragments.

In order to further study the interaction of tumor cells with laminin, a glycoprotein of basement membranes, Dr. Liotta's group identified the domain of the laminin molecule which attached to the tumor cell. In addition, a specific cell membrane receptor for laminin was identified by Dr. Liotta's group for the first time. By rotary shadowing electron microscopy, performed by Ms. Margulies, laminin has the configuration of a cross with 3 short arms and one long arm. The cell binding site resides near the intersection of the short arms. The type IV collagen binding domain of laminin is associated with the globular end regions of the short arms but not the long arm. The long arm contains a major binding site for basement membrane proteoglycan. The laminin receptor has a Kd of 2×10^{-9}, 10-100,000 receptors per cell. The receptor is present on isolated cell membranes and is destroyed by trypsin. The receptor was isolated from cell membranes by detergent treatment, HPLC, nitrocellulose blotting and/or laminin affinity chromatography. The laminin receptor molecular weight is 67,000. A fragment of the laminin molecule which binds to the receptor and blocks attachment will inhibit metastases formation in animal models. Laminin receptors can be readily measured in surgical specimens of human breast cancer tissue. Monoclonal antibodies have been prepared to the human laminin receptor.

The interaction of the tumor cell with its extracellular matrix may play an important role in determining its metastatic and invasive properties. To better understand the protein components that make up the extracellular matrix and how they are regulated, this section has undertaken to construct, isolate, and characterize molecular clones of laminin receptor and of several different collagens. Using a monoclonal antibody directed against the laminin-binding domain of laminin receptor, we screened a human endothelial cell cDNA λgtll library. Six plaques showed an intense reaction with the antilaminin receptor monoclonal but showed no reactivity toward a variety of control antibodies. The sizes of the cDNA inserts of the six clones ranged from 450 to 950 base pairs. Restriction enzyme mapping and Southern hybridization identified a 400 base pair fragment which was identical in each cDNA clone, suggesting that this fragment may represent the laminin-binding domain of laminin receptor. The putative laminin receptor cDNA clone recognizes a 1700 base mRNA, which would be sufficient in

length to code for a protein with the expected size of laminin receptor. In addition, cDNA clones coding for mouse type IV collagen and genomic clones coding for human type II collagen have been characterized.

Type IV collagenase is an important basement membrane degrading metalloproteinase that malignant cells produce and secrete to facilitate their traversation through blood vessel walls as well as during transition from in situ to invasive carcinoma. Human type IV collagenase was isolated from culture supernatants of A2058 metastatic melanoma cells after four steps of purification. The collagenase has a molecular weight of 70 KDa and produced a typical cleavage pattern of type IV collagen by SDS gel electrophoresis. A second polypeptide was eluted off the collagen IV affinity column which has a molecular weight of 100 KDA. This new collagen IV binding protein has no collagenolytic activity and does not possess any characteristics of known matrix proteins.

This section is studying biochemical events characteristic of malignant tumor cells, which must be highly motile while invading tissue and metastasizing to distant sites. They have found that a number of metastatic cell lines produce and respond to autocrine motility factors. A partially purified material from the conditioned media of a human melanoma cell line was found to be a protein with an Mr of about ~ 55 KD. The material induces a strong chemotactic response in the producer cells and appears to exert its action by perturbing membrane phospholipid metabolism of the cell. They have extended these observations to 3T3 cells and their transformed metastatic counterparts. They find that the transformed cells produce and respond well to autocrine factors but poorly to platelet-derived growth factor (PDGF). The nontransformed cells, on the other hand, respond well to PDGF but to a lesser extent to the autocrine factors of the transformed cells. The nontransformed cells do not produce autocrine motility factors. These results suggest that an important characteristic of the metastatic phenotype of malignant cells is their ability to produce and respond to autocrine motility factors.

This section is investigating the molecular biology of tumor metastasis and invasion. They are using a variety of techniques to identify specific genetic elements whose expression is altered in metastatic cells. Pulse-labeling studies of paired nonmetastatic-metastatic cells revealed differences in the synthesis of specific proteins. RNA from cultured cell lines and tissues with varying metastatic potential have been analyzed by cell-free translation in a rabbit reticulocyte lysate and by hybridization analysis. In vitro translation studies indicated that the levels of several specific mRNAs are either markedly increased or decreased in metastatic murine melanoma cells. A cDNA library of the murine melanoma cells was constructed. Approximately 40,000 clones of the recombinant DNA library were screened to isolate specific genes involved in the etiology and maintenance of the neoplastic state. To date, they have isolated several molecular clones which code for mRNAs which are expressed to a differential degree in metastatic versus nonmetastatic lines. In addition, levels of mRNA for the major excreted protein (MEP) of transformed murine cells are increased in the nonmetastatic cells. In contrast, levels of mRNA for type IV collagen are increased in the metastatic cells.

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The ability of ras^H genes to induce metastatic potential as well as transformation and tumorigenicity was demonstrated in several cell types. NIH-3T3 cells transformed with either the DNA of the Harvey Sarcoma Virus or with the cloned T24 human ras^H oncogene resulted in cells which formed lung metastases. Early passage diploid rat embryo fibroblasts transformed by ras^H (Pozzatti et al) were also metastatic as were early passage Chinese hamster lung fibroblasts transformed by ras^H (Spandidos and Wilkie, Nature (1984) 310: 469). Thus, the ras^H oncogenes can induce metastatic behavior even in diploid fibroblasts.

However, transformation itself or the ability to grow as a tumor was shown to be insufficient to result in metastasis. The normal cellular counterpart of the ras^H oncogene can also transform NIH-3T3 cells if an LTR is placed upstream from the c-ras^H gene to increase the levels of the normal P21 (Chang et al, Nature (1982) 307: 658). The NIH-3T3 cells transformed by this construction, while highly tumorigenic, do not metastasize.

Ras^H did not induce metastatic potential in all recipient cell lines tested. For example, C127 cells, a murine epithelioid line which were transformed by ras^H, did not metastasize although morphologically transformed and highly tumorigenic. The induction of metastatic potential in those cells is being attempted in gene transfer experiments.

F. Hematopathology Section

The Hematopathology Section conducts a major program in diagnostic and experimental hematopathology. Drs. Jaffe and Cossman are board-certified pathologists who take responsibility for diagnostic hematopathology for patients admitted to the Clinical Center. While most of this material pertains to patients with malignant lymphoma admitted to the clinical services of the National Cancer Institute, collaborations are also conducted with NIAID and NIAMDD for patients with reactive lymphoproliferative lesions. Drs. Jaffe and Cossman also receive several hundred cases submitted for diagnostic consultation from pathologists in the regional medical community as well as throughout the United States and other parts of the world.

The Hematopathology Section continues its active research program on the immunological characterization of malignant lymphomas. All patients with newly diagnosed lymphomas or recurrences are studied for phenotypic and functional markers. This information is utilized to study the relationship of malignant lymphomas to the normal immune system, to develop improved classifications of disease, and to distinguish new clinicopathologic entities. This information is also being used as a basis for immunotherapy in collaboration with the Medicine Branch, DCT, and the Biological Response Modifier Program in Frederick, Maryland, NCI. Based on the studies of the Hematopathology Section, a classification of post-thymic T-cell malignancies has been proposed. This classification delineates at least five major clinicopathologic entities with differing clinical presentations and prognoses.

Dr. Charles R. Simrell, a Fellow in the Hematopathology Section, published a study which demonstrated lymphokine-induced phagocytosis in the angiocentric immunoproliferative lesions (AIL) (lymphomatoid granulomatosis and related

disorders). Phagocytosis-inducing (PIF) activity was demonstrated in patients with AIL but not with other peripheral T-cell neoplasms, B-cell lymphomas, or Hodgkin's disease. Moreover, patients in both the "benign" and "malignant" phases of their disease showed comparable PIF activity. This observation would suggest that AIL represents a single nosologic entity, and that "conversion" to lymphoma does not occur. The lymphokine identified differs from γ interferon, macrophage activating factor, and other recognized lymphokines, and is derived from a T4 positive cell.

A functional study of follicular non-Hodgkin's lymphomas showed that the neoplastic cells in virtually all cases could be induced to secrete monoclonal immunoglobulin. However, induction of immunoglobulin secretion required the addition of allogeneic helper T lymphocytes. T cells derived from involved lymph nodes were not capable of mediating such help, and in fact, appeared to exert a suppressive effect for immunoglobulin secretion in in vitro culture. Another study demonstrated that in some patients with follicular lymphoma, (a monoclonal B-cell tumor), mature T cells were numerically predominant. However, morphologically and phenotypically the infiltrating T cells appeared to be non-neoplastic. The infiltrating T cells may represent a positive host immune response, since the patients continued to pursue an indolent clinical course, even with apparent histologic progression. Both of these studies suggest that the neoplastic cells of follicular lymphoma are susceptible to host immunoregulation.

Dr. Cossman initiated a new research program over the past year regarding the molecular basis of the diagnosis of human lymphoproliferative disease. This work has already produced a number of major findings. The T- and B-cell lineage of diffuse, aggressive lymphomas as determined by phenotypic studies can be confirmed by detection of appropriately rearranged T-cell receptor or immunoglobulin genes. Moreover, in some patients' tissues which lack evidence of malignant lymphoma by conventional histology or immunologic studies, malignant lymphoma can be identified by these techniques. Molecular genetic analysis has also identified that a subclass of diffuse, aggressive lymphomas contain the t14,18 chromosomal translocation characteristic of follicular lymphoma. This observation suggests that many of these high grade B-cell lymphomas may originate as follicular lymphomas both clinically and at the molecular level.

Precursor T-cell neoplasms were shown to occupy sequential differentiation compartments during early T-cell development as shown by the coordinate expression of surface membrane molecules and T-cell receptor rearrangements. Monoclonality and T-cell lineage were also demonstrated by rearrangement of the T-cell receptor gene in Tγ lymphoproliferative disorder, a disorder in which a neoplastic basis had been questioned. Angio-immunoblastic lymphadenopathy, currently considered an atypical reactive process with a high incidence of progression to malignant lymphoma, in all cases was shown to contain monoclonal rearrangements of either immunoglobulin genes, T-cell receptor genes, or both simultaneously.

Although in most patients with malignant lymphoma, sequential studies show clonal fidelity by these analyses, in follicular B-cell lymphomas there is a

high frequency (35%) of clonal evolution. Such clonal evolution allows for the escape of the malignant cells from identity by anti-idiotypic antibody. In one case, alteration of idiotype was likely attributable to somatic mutation of a V region gene. Other mechanisms of clonal evolution may include new variable gene rearrangements and heavy chain constant region isotype switching. Deduced geneologies of the related cloned argue for an immature follicular lymphoma progenitor cell in which immunoglobulin genes remain in a germline configuration. Although new rearrangements continue to occur in the productive allele, there is conservation of the immunoglobulin allele involved in the t14,18 translocation characteristic of follicular lymphomas. These observations have important significance for treatment strategies for the follicular lymphomas which, although clinically low-grade, have been refractory to cure with current regimens. Moreover, the capacity of follicular lymphomas to frequently alter their surface membrane idiotype may allow them to escape detection by the host immune response and may account for the clinically observed waxing and waning clinical course of disease.

Dr. Neckers participates in the clinical work of the Hematopathology Section by supervising the cell sorter used in clinical phenotypic studies. Dr. Neckers also conducts an active research program on the regulation of cell growth by transferrin receptors. Major findings include the fact that normal cells regulate transferrin receptor appearance by carefully controlled tissue-specific growth factors and their receptors. However, in malignant cells this regulation is lost and transferrin receptor expression becomes constitutive. Nevertheless, both growth factor dependent and constitutive transferrin receptor expression is calcium dependent while transcription of the IL2 receptor gene and genes for C-myc and C-myb are not calcium dependent. Transferrin receptor redistribution and its associated hyperphosphorylation can be dissociated by a blockade of calcium channels. Hyperphosphyorlation is not calcium dependent but redistribution is and may reduce surface receptor levels by 60% without affecting proliferation. Cyclic AMP terminates transferrin receptor gene transcription in normal and some malignant cells and is correlated with a G1 arrest.

Dr. Holbrook, a Senior Staff Fellow in the Hematopathology Section, has continued her work on the control of the interleukin 2 gene in normal and malignant cells. She has succeeded in stably transfecting monkey and mouse fibroblasts with fusion genes containing the IL2 cDNA expressed under the control of the mouse metallothioneine and SV40 by fibril promoters. IL2 is produced in these stable transformants. She has also shown for the first time the production of IL2 mRNA in an *in vivo* situation. In the monkey, following antigenic stimulation with complete Freund's adjuvant, IL2 mRNA can be demonstrated. Furthermore, it has been shown that sequences in the 5' flanking region of the IL2 gene share limited homology to HTLV-I which, like IL2, is T-cell tropic. In collaboration with Dr. Yuan Devries (E.I. duPont, Glenholden, PA), she has shown that in MLA-144 cells, a Gibbon T-cell line that constitutively produces IL2, the IL2 gene is rearranged. At least part of the rearrangement is due to an insertion of the LTR of the gibbon ape leukemia virus at the 3' end of the gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00853-32 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Surgical Pathology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: E.E. Lack Chief, Surgical Pathology & Postmortem Section LP, NCI
OTHER: (see next page)

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology & Postmortem Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
20	20	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Surgical Pathology and Postmortem Section, together with the Cytopathology Section, Ultrastructural Pathology Section and Hematopathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all institutes in those investigations which involve the use and study of human pathological material. A new frozen section and surgical pathology processing area was constructed adjacent to the new operating rooms and has been in use since April, 1983. This new facility has greatly enhanced processing of specimens and communication of diagnostic findings with attending physicians. It is equipped with intercom and television viewing screens in each operating room to facilitate communication.

The staff is actively engaged in a variety of projects involving clinicopathological correlation and pathologic characterization of diseases studied at the Clinical Center. Up-to-date immunocytochemical techniques have been applied to the study of tumors and other non-neoplastic diseases. The use of immunohistochemical staining has greatly facilitated more precise diagnosis in selected cases and with the increasing number of monoclonal antibodies available this technique should have even greater value in diagnostic and research pathology. A major renovation of the histology laboratory is in the planning stage. This will include space allocated for performance of special stains and immunocytochemistry. Renovation of the autopsy facility is essentially complete and its use has greatly enhanced the service.

PHS 6040 (Rev. 1/84) GPO 914-918

Other Professional Personnel:

*T.J. O'Leary	Acting Chief, Autopsy Service	LP, NCI
C.M. Reichert	(Formerly) Chief, Autopsy Service	LP, NCI
+D.L. Levens	Medical Staff Fellow	LP, NCI
+C.C. Baker	Medical Staff Fellow	LP, NCI
+K. Bernstein	Medical Staff Fellow	LP, NCI
+M.R. Lieber	Staff Fellow	LP, NCI
+S.-H. Lo	Staff Fellow	LP, NCI
+A.M. Macher	Sr. Surgeon	LP, NCI
+S.M. Mackem	Staff Fellow	LP, NCI
+M.A. Thompson	Staff Fellow	LP, NCI
+E.H. Radany	Staff Fellow	LP, NCI
G.J. Bryant	Expert	LP, NCI
C.R. Simrell	Jr. Staff Pathologist	LP, NCI
S.E. Martin	Staff Pathologist	LP, NCI
E.S. Jaffe	Chief, Hematopathology Section	LP, NCI
T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI
J. Cossman	Senior Investigator	LP, NCI
C. Restrepo	Expert	LP, NCI
E. Lipford	Expert	LP, NCI
*L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP, NCI
D.A. Katz	Senior Staff Fellow	OCD, NINCDS
*K.J. Stromberg	Consulting Pathologist	LVC, NCI
*W.C. Roberts	Chief	IRPA, NHLBI
*M. Valsamis	Visiting Consultant - Bird Coler Hospital, New York, NY	

Project Description

Objectives:

The objectives of the Surgical Pathology and Postmortem Section are: (1) to provide diagnostic services in pathologic anatomy to the clinical research projects conducted at NIH; (2) to carry out independent research; (3) to provide a residency program in anatomic pathology; and (4) to collaborate with investigators in research involving the use and study of human materials.

The service functions of the section during the past year included:

(a) 153 autopsy examinations. The residents perform nearly all of the postmortem dissections under supervision of various staff members. A conference is held weekly by the staff and residents where gross organs are reviewed and clinical pathologic correlations are made. The residents review the microscopic slides from each autopsy they perform with one of the staff before completing the autopsy protocol.

The staff assists the residents in preparing for the numerous clinical conferences in which the section participates.

+These physicians are full-time Residents in the Laboratory of Pathology.
*These Associate Pathologists spend part time in the activities of the Surgical Pathology and Postmortem Section.

(b) Approximately 5500 specimens were accessioned in the Surgical Pathology Section. The surgical pathology specimens are initially examined and prosected by a resident and their reports are checked by the staff. Associate pathologists from other laboratories or sections are frequently consulted about diagnostic problems in surgical pathology and/or assume responsibility for handling certain tissue removed for specific research projects. Approximately 600 consults were rendered.

(c) Histological preparation and special procedures: The functions are carried out under the direction of Mrs. Ruby Howard. More than 53,000 slides were prepared. Of these, 4/5 were routine H&E stained and 1/5 were specially stained. The laboratory is very active in specialized immunocytochemistry.

(d) Photographic services of the department: A photographic record is made of the large amount of pathological material which is handled and studied in the department. The photography is done by the staff and residents with the technical assistance of Mr. J. Banks who also maintains the equipment and helps with much of the photographic processing. Gross photographs and photomicrographs of the pathologic material are provided to the clinical staff on request, and are used extensively for conferences and various seminars. Many are prepared for publication. Over 325 black and white negatives and over 640 black and white prints have been prepared from autopsy and surgical pathology material. More than 50 35 mm rolls have been prepared from gross specimens seen on the surgical and autopsy service.

A large proportion of the photomicrography is done under the direction of Mr. Ralph Isenburg, who provides professional assistance and facilities for the entire staff of the Laboratory of Pathology.

Conferences. The staff takes part in the following inter-departmental conferences:

Pediatric Oncology Tumor Board (weekly)
Sarcoma Staging Conference (weekly)
Surgical Morbidity and Mortality Conference (monthly)
Medicine-Radiation Conference (weekly)
Surgical Pathology Conference (weekly)
Lymphoma Staging Conference (weekly)
Testicular Tumor Staging Conference (weekly)

In addition, the staff participates in clinicopathological conferences discussing specific patients.

Data retrieval system. In conjunction with DCRT, a program has been created for storing and retrieving the surgical pathology and autopsy material and to automatically encode all the diagnoses. The language used is a modification of SNOP and a dictionary is being constantly updated in order to accommodate a maximum of currently used terms.

Proposed Course of Research:

(a) Continue to provide the services described. (b) Increase the interaction with the clinical branches in the design and evaluation of protocols. (c) Improve the opportunities for the resident staff to participate in teaching, conferences, and seminars, and provide elective periods to be spent accomplishing research projects with the senior staff. (d) Implement data retrieval programs.

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Bernstein, K., Alexander, C.B., Reddy, P., and Mage, R.G.: Complete sequence of a cloned cDNA encoding rabbit secreted μ chain of V_H a2 allotype: Comparisons with V_H a1 and rabbit membrane μ sequences. J. Immunol. 132: 490-495, 1984.

McCartney-Francis, N., Skurla, R.M., Jr., Mage, R.G., and Bernstein, K.: Kappa chain allotypes and isotypes in the rabbit: cDNA sequences of clones encoding b9 suggest an evolutionary pathway and possible role of the inter-domain disulfide bone on quantitative allotype expression. Proc. Natl. Sci. USA (in press)

Bernstein, K., Lamoyi, E., McCartney-Francis, N., and Mage, R.G.: Sequence of a cDNA encoding Basilea Kappa light chains (K2 isotype) suggests a possible relationship of protein structure to limited expression. J. Exp. Med. 159: 635-640, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09126-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Measuring malignancy of human cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: C.H. Fox Senior Scientist LP, NCI

COOPERATING UNITS *(if any)*

Inst. for Histol., Karolinska Inst., Stockholm, Sweden; Dept. of Cytopathol., Karolinska Hosp., Stockholm; Dept. of Electrical Engineering & Computer Sci., Univ. of Linkoping, Sweden; Dept. of Elect. Engineer., Carnegie-Mellon Univ.

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology and Postmortem Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project develops objective measurements of the biological malignancy of human cancers with the intention that clinicians shall have better information bases for planning treatment. There are several discrete goals: 1) To develop better methods for preparing histological material for pathological examination so that objective criteria may be applied, 2) To identify those properties of tumor material that have relevance to the biological behavior of cancer based on prospective and retrospective studies, 3) To study the mechanisms by which malignancy is manifested such as invasion and metastasis using both human tissues and isolated cell populations. The methods used in this study include stoichometric histochemistry, quantitative light microscopy, computerized image analysis, confocal laser microscopy, and computerized population register epidemiology.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

Estimating the malignant potential of human cancers of epithelial and mesothelial tissues has traditionally been entirely subjective. This project uses objective measurements of tissue components to add information to the decision "tree" used in estimating prognostic potential of cancers. Recent advances in computer technology and optical design have converged to now make it possible to process the enormous amount of data and biological information so that it has practical consequence for understanding cancer biology. The objective then of this research is to provide information about the biological characteristics of a specific tumor that can be used to estimate the aggressiveness of that tumor. Clinical decisions may then be made as to the aggressiveness of therapy.

Methods Employed:

The methods employed will be described under each study heading.

Study 1. Morphological pathology is based on recognition of two dimensional patterns produced as consistent artifacts. The cognitive processes used by the pathologist are highly complex based usually on decision tree processes with deductive steps. Other side branches of decision making are based on a relatively few objective components such as staining reactions, antibody reactivity and estimated values such as mitoses per field. The technical aspects of preparing specimens for examination for morphological pathology are geared toward selecting diagnostically promising pieces or areas of tissue or even portions of the specimen that are "representative" of an organ or lesion. The pathologist greatly reduces the amount of tissue to be examined by this process. Objective criteria of specimen selection or statistically cogent sampling does occur, but is unusual. After sample selection by gross appearance, the tissue is subjected to preparatory techniques that ultimately result in actual slides, but only after solvent extraction, heat treatment and staining with relatively crude histological stains. Preparing slides further reduces the actual amounts of tissues examined by the pathologist under the microscope. In most cases, only about 1×10^{-3} of the tissue block is examined by the pathologist which means that tissues for examination may represent as little as 1×10^{-4} or less of the actual specimen unless the submitted tissue is by nature tiny, such as in many biopsies. Study 1 is concerned with improving this process. Tissues undergo a variety of changes while being processed. The most obvious uncontrolled variable is ensuring the stability of form of the tissue during fixation. Since most tissues used in pathology are fixed in formaldehyde, a somewhat involved study of the chemistry and reactions of formaldehyde has been conducted. Aqueous formaldehyde has the unusual property of containing only small amounts of formaldehyde not bound as methylene glycol. The behavior and reactivities of formaldehyde in aqueous solutions has been reported in a comprehensive treatise (1). Other aspects of histological preparation such as sampling error in tissue selection for sectioning are continuing.

Study 2. Analysis of histological preparations to improve histological grading: A new dimension in pathology. Traditionally, histological preparations have been studied as two dimensional images. A stained slide has very little depth and the pathologist must imagine the structure and organization of a tissue or organ based on his or her understanding of anatomy and the orientation of the tissue. Attempts at analysis of images the pathologist uses in his or her decision making is limited by the number of parameters that can be measured and the ease with which they can be measured. Recently morphometry has been popular, based on the measurements used by Chalkley at NCI in the 1940s. The advent of array processors and interactive image analysis software have greatly improved the ease with which such determinations may be made, but they are still very time consuming and show little promise for being effective in general use.

An alternative that may revolutionize histopathology is the simultaneous development of the confocal laser-microscope and very large image memory devices. This development is the result of my collaboration with the image analysis group of Linkoping University and the Department of Pathology of the University of Uppsala. A laser beam is focused in space to a point about 0.5 μm in volume. The specimen is moved over the focused coherent light and the fluorescence of the fluorochrome nucleus is recorded. The entire object is scanned at a single level of focus and the image is stored. The focus is moved 0.5 μm deeper into the sample and scanned again. This process is repeated through the entire specimen thickness. Using an extremely powerful computer, the two dimensional images are assembled into a three dimensional image and presented on an appropriate 3-D display monitor. Since the image has been digitized, it is relatively easy to convert the data from cell fluorescence into quantitative values for each cell, nucleus, or element. This technology allows simultaneous quantitative determinations such as DNA values for tumor cell populations, but also allows an understanding of invasion and metastasis at the cell-tissue level. The technical difficulties so far have been limited to development of optical methods of removing dispersive and refractive background elements. In conjunction with this study, we are also developing a very high speed prescreening machine for population screening from cervical cancer. Current progress shows a machine that produces a reasonable level of false positives with no false negatives, using conventional Pap smears.

Study 3. As an ongoing part of studies of malignancy, I have been conducting basic research on the cellular mechanisms of cell attachment and the anatomical differentiation of cells in culture. Using a relatively new microscopic technique, it is possible to observe the attachment of cells to substrates and to follow the steps in "differentiation" of the cytoskeleton. Cultured cells attach to a new substrate or to other cells by a very involved process. Adhesion is followed by synthesis of focal contacts that in turn involve synthesis of structural fibers of the cytoskeleton. Many differentiated normal cells seem unable to dissassemble their cytoplasm once it is formed, but most malignant cells seem capable of doing so.

Significance to Biomedical Research and the Program of the Institute:

Pathology is remarkably reliable in diagnosis of most cancers. The question of whether a particular slide shows the presence of cancer has been solved through over a century's experience in subjective image analysis and pattern recognition. On the other hand, the biological behavior of the cancer after diagnosis is far more problematical. Grading schemes used to estimate malignancy show surprising lapses of reliability and since the advent of large varieties of treatment modalities of differing degrees of toxicity, such determinations have increasing importance. The aim of this research is to provide further information to the clinician in choosing treatment. The National Cancer Institute has been outstanding in developing new treatment modalities; these projects are designed to facilitate selection of treatments appropriate to the gravity of the disease.

Publications:

Fox, C.H., Johnson, F.B., and Roller, P.: Fixation by formaldehyde. J. Histochem. Cytochem. (in press)

Stenkvist, B., Bergstrom, R., Eklund, G., and Fox, C.H.: Papanicolaou smear screening and cervical cancer - What can you expect? JAMA 252: 1423-1426, 1984. (Translated into six other language editions)

Stenkvist, B. and Fox, C.H.: The doctor's dilemma: False negative tests. Lancet (in press)

Fox, C.H.: Prudent practices. J. Histotech. 7: 119-120, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09144-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Identification of sequence specific DNA binding proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: D.L. Levens

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology and Postmortem Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2/3	2/3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have developed a general method for the enrichment and identification of sequence specific DNA binding proteins. A well-characterized protein-DNA interaction is used to isolate proteins from crude cellular extracts or fractions thereof, which bind to specific DNA sequences based solely on this property. The DNA sequence of interest, cloned adjacent to the lac operator DNA segment is incubated with a lac repressor-beta-galactosidase fusion protein which retains full operator and inducer binding properties. The DNA fragment bound to the repressor-beta-galactosidase fusion protein is precipitated by the addition of affinity purified anti-beta-galactosidase immobilized on beads. This forms an affinity matrix for any proteins which might interact specifically with the DNA sequence cloned adjacent to the lac operator. When incubated with cellular extracts in the presence of excess competitor DNA, any protein(s) which specifically binds to the cloned DNA sequence of interest can be cleanly precipitated. Upon addition of IPTG, the lac repressor releases the bound DNA and thus the protein-DNA complex consisting of the specific restriction fragment and any specific binding protein(s) is released, permitting the identification of the protein by standard biochemical techniques. We demonstrate the utility of this method by using the lambda repressor, another well characterized DNA binding protein as a model. In addition, using crude preparations of the yeast mitochondrial RNA polymerase, we have identified a 70,000 MW peptide which binds specifically to the promoter region of the yeast mitochondrial 14S rRNA gene.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

1. To develop a general method for the identification of sequence specific DNA binding proteins which are the mediators of the essential processes such as transcription, recombination and replication.

2. Application of this technique to study transcription in yeast mitochondria as a model system to verify the validity of the approach.

3. Application of the technique to study factors which regulate the expression of genes regulated by the general control of amino acid metabolism in yeast nuclei.

4. Extension of the method to study tissue specific and/or inducible gene expression in mammalian cells to identify factors which regulate transcription.

Methods Employed:

See above.

Major Findings:

A method has been developed which allows the knowledge of cis-regulatory DNA sequences to be used to identify and enrich protein components which interact with those sequences. The feasibility of the technique has been verified by demonstrating that the lambda repressor can be isolated in one step from whole cell extracts of E. coli. The technique has been applied to yeast mitochondria and has demonstrated the existence of a protein factor which binds to promoter regions of mitochondrial genes.

Significance to Biomedical Research and the Program of the Institute:

The regulation of gene expression is a central problem in molecular biology. Recombinant DNA technology and efficient transfection procedures have allowed the definition of DNA sequences which regulate vital physiological processes; this technique allows the identification of some mediators of these processes which could potentially greatly increase our understanding of gene regulation. Because aberrant gene expression is increasingly recognized as an important contributor to pathologic events, including neoplasia, a knowledge of mechanisms of transcriptional regulation is necessary for a complete understanding of many disease processes.

Proposed Course of Research:

To apply the newly developed method to study tissue specific and inducible gene expression.

Publications:

Levens, D. and Howley, P.M.: A novel method for identifying sequence specific DNA binding proteins. Mol. Cell. Biol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09145-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Neuropathology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: D.A. Katz	Neuropathologist	OCD, NINCDS

COOPERATING UNITS *(if any)*

Surgical Pathology and Postmortem Section, LP, NCI

LAB/BRANCH

Office of the Clinical Director, NINCDS

SECTION

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Subspecialty expertise in diagnostic neuropathology is provided to the Laboratory of Pathology, NCI, and to all other institutes, via the Office of the Clinical Director, NINCDS. The neuropathology service is integrated with the Surgical Pathology and Postmortem Section, and also, more recently, with the Ultrastructural Pathology Section. The service also functions in a collaborative manner to provide neuropathological expertise in a variety of clinicopathologic investigations.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

The objectives of the neuropathology service parallel those of the Surgical Pathology and Postmortem Service: (1) to provide a specialized diagnostic service for neurosurgical and autopsy material from NIH patients; (2) to use this material to carry out clinicopathologic studies of primary neurologic disease and neurologic complications of systemic disease, and to (3) teach resident trainees in anatomic pathology the fundamentals of neuropathology; (4) to assist, in collaborative fashion, basic investigators who desire to study human nervous tissue.

Autopsy: 153 autopsies were performed at NIH in the last year; the brain was examined in approximately 100 cases, and at least 25% of these manifested significant primary or secondary neurologic findings. Neuropathologic consultation is available at the time of autopsy, as needed, for special handling of the brain and/or spinal cord. Detailed and standardized gross examination, description and photography are carried out with the pathology residents at weekly brain cutting sessions. The microscopic slides of all brains and spinal cords are reviewed by the neuropathologist, and the findings integrated into the autopsy report. Presentations of pertinent findings at gross autopsy conference and other clinical conferences are performed by the resident in consultation with the neuropathologist.

Surgicals: Approximately 175 neurosurgical specimens are examined yearly, including both submitted and in-house cases. Approximately 35 intra-operative frozen section consultations are provided yearly.

Conferences: In addition to those mentioned above, both lectures and formal neurologic CPC's (clinicopathologic conferences) are given.

Proposed Course of Research:

Incorporation of the neuropathology material into the data retrieval system will allow for improved resident teaching, with creation of a separate teaching file, and will facilitate restrospective case studies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00852-32 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Exfoliative cytology applied to human diagnostic problems and research problems

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E.W. Chu	Chief, Cytopathology Section	LP, NCI
OTHER:	S.E. Martin	Staff Pathologist	LP, NCI
	Y. Ye	Visiting Fellow	LP, NCI
	S. Moshiri	Visiting Fellow	LP, NCI
	T.A. Wood	Biologist	LP, NCI
	L. Galito	Biologist	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8	4	4

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Cytopathology Section provides complete diagnostic service in exfoliative cytology, medical cytogenetics, and fine needle aspiration cytology. The section has also initiated application of immunocytochemistry techniques to improve and enhance cytological diagnostic efficacy. In addition, the section collaborates in various clinical research projects utilizing special techniques including special staining, tissue culture techniques, as well as investigating chromosomal and/or somatic cell hybridization techniques in mapping genes.

PHS 6040 (Rev. 1/84) GPO 914-916

Other Professional Personnel:			
	A.M. Wilder	Biologist	LP, NCI
	K. Nakahara	Biologist	LP, NCI
	E. Sanders	Bio. Lab. Tech.	LP, NCI

Project Description

Objectives:

1. To provide the staff of the Clinical Center with an accurate and complete exfoliative and aspiration cytology service as well as diagnostic cytogenetics (karyotyping and *in situ* hybridization).
2. To collaborate in various clinical research projects evaluating cancer therapy, the hormonal status of the cancer patients, the course and natural history of the cancer lesions and the anatomical and physiological changes in the human body associated with various pathologic conditions.
3. To collaborate with the Dental Research Institute in the evaluation of intra-oral fluoride releasing device by examination of oral epithelial cells.
4. To collaborate with interdepartmental investigators in the cytogenetic studies of human progression and metastatic lesions.
5. Development of better diagnostic techniques.

The specific objectives include:

A) Cytology

1. Continued improvement in cytologic techniques in establishing definitive diagnosis.
2. Improvement in fine needle aspiration cytologic technique.
3. Establishing cytologic characteristics of cell types seen only in fine needle aspirations.
4. Setting up special stain techniques which may be used for general cytology or special research problems.
5. To use immunoperoxidase technique on cytological material to enhance the establishment of definitive diagnoses.
6. To use Pap stain on oral smears from patients who are fitted with the intra-oral fluoride device.
7. Maturation index in precocious children.

B) Diagnostic Cytogenetics

1. Improvement in short-term culture system to assure of successful harvesting of *in vitro* cells.
2. Improvement in staining techniques.
3. Improvement in special chromosomal analytical techniques such as banding by various methods.
4. Gene mapping by chromosome *in situ* and somatic cell hybridization.

C) Research Cytogenetics

1. Chromosomal characterization of oncogene transformed rat cell lines.
2. Cytogenetic studies of metastases (animals).
3. Chromosomal characterization of purified (Hodgkin's) Reed-Sternberg cells.

Methods Employed:

1. Special procedures in addition to the standard Papanicolaou stain are used:

    A. Millipore filtration of and cytocentrifuge all fluids
    B. Special stains
        1. Feulgan reaction for sex chromatin bodies
        2. Acid fast, Pneumocystis, Methenamine silver, Brown Brenn for various organisms and fungi
        3. PAS, Wright Giemsa, iron stain, melanin stain, congo-red, oil-red-O, mucicarmine for various specific reactions

2. Short-term cultures of peripheral blood, bone marrow cells and tumor cells found in body fluids are the standard diagnostic methods.

In addition, special techniques involving trypsin digestion followed by special stains such as G-banding, Q-banding, and C-banding, as well as fluorescence staining whenever indicated. Also, somatic cell hybridization and autoradiography are performed whenever needed.

Major Findings:

1. Providing prompt services and early diagnosis in cytological materials.
2. Providing cytologic evaluation and estimated ranges of either relapse or remission in the continual management of meningeal leukemia.
3. Providing cytological evaluation of therapeutic effects on cells seen in urinary specimens, vaginal-cervical smears, sputum and effusions.
4. Providing chromosomal analysis for clinical studies as well as in the establishment of definitive malignancies.
5. By using chromosomal and/or somatic cell hybridization techniques, certain immunoglobulin genes were mapped to sites of non-random chromosomal translocations, e.g. in malignant B-cell lymphoma.
6. Providing cytologic methods to research projects; specifically, fine needle aspiration in thyroid nodules.
7. Immunohistochemical technique often offers means to differentiate reactive cells from cancer cells in body fluids by using suitable antisera.

Significance to Biomedical Research and the Program of the Institute:

1. The diagnostic value of exfoliative cytology in the clinical management of various disease states is an established fact.
2. Ability to discern specific therapeutic effects is crucial in patient (therapy) management.

3. Aspiration cytologic techniques are useful in establishing diagnosis on metastatic diseases and/or recurrent malignancies. In such instances, the fine needle aspiration technique eliminates the more involved incisional biopsy and therefore is time saving and more economical to the clinicians as well as to the patients.
4. Immunocytochemical techniques may offer early resolutions to equivocal cases.
5. Immunoperoxidase technique can support the clinical diagnosis of malignancy with specific monoclonal antibodies.
6. Chromosomal analysis is a way of establishing definitive diagnoses in selected patients with endocrine disorders, congenital defects and also in some malignancies.
7. Cytogenetic studies abet and confirm the ongoing research work on tumor progressions.
8. Chromosomal and/or somatic hybridization techniques will eventually open the way for extensive experimental tests involving genes.

Proposed Course of Research:

1. Continue to provide a complete cytodiagnostic service for the entire staff of the Clinical Center in various research projects.
2. Continual improvement and development of established as well as new techniques in the cytological field.
3. Continual work with gene mapping by hybridization technique.
4. Develop and improve prophase banding technique.
5. Intensive pursuit of immunocytochemical techniques to improve and abet diagnostic cytology.

Activities:

1. Seminar on Fine Needle Aspiration Cytology, Department of Pathology, Providence Hospital, 1984.
2. Vice President - Chinese Medical and Health Association.

Publications:

Whang-Peng, J., Knutsen, T., Douglass, E.C., Chu, E.W., Ozols, R.F., Hogan, W.M., and Young, R.C.: Cytogenetic studies in ovarian cancer. Cancer Genet. Cytogenet. 11: 91-106, 1984.

Chu, E.W. and Martin, S.E.: Fine needle aspiration cytology of breast. Indian J. Cytol. 1: 70-78, 1984.

Grimm, E.A., Vose, B.M., Chu, E.W., Wilson, D.J., Lotze, M.T., Rayner, A.A., and Rosenberg, S.A.: The human mixed lymphocyte-tumor cell interaction test. I. Positive autologous lymphocyte proliferative responses can be stimulated by tumor cells as well as by cells from normal tissues. Cancer Immunol. Immunother. 17: 83-89, 1984.

Martin, S.E., Zhang, H.-Z., Magyarosy, E., Jaffe, E.S., Hsu, S.-M., and Chu, E.W.: Immunological methods in cytology: Definitive diagnosis of non-Hodgkin's lymphomas using immunological markers for T and B cells. Am. J. Clin. Pathol. 82: 666-673, 1984.

El-Bolkainy, M.N., Chu, E.W., Ghoneim, M.A., Raafat, M.M., Tawfik, H.N., El-Morsey, B.A., Aboul-Ela, F., Dahaba, N.M., and Ibrahim, A.S.: Cellular dysplasia in urine: Cytologic follow-up study on 220 cases. J. Egyptian Cancer Inst. 1: 13-23, 1984.

Rayner, A.A., Grimm, E.A., Lotze, M.T., Chu, E.W., and Rosenberg, S.A.: Lymphokine-activated killed (LAK) cells. Analysis of factors relevant to the immunotherapy of human cancer. Cancer 55: 1327-1333, 1985.

Ramacciotti, C.E., Pretorius, H.T., Chu, E.W., Barsky, S.H., Brennan, M.F., and Robbins, J.: Diagnostic accuracy and utility of aspiration biopsy of thyroid nodules. Ann. Intern. Med. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00897-02 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cytological diagnosis of lymphomas by immunocytochemistry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S.E. Martin	Surgeon	LP, NCI
OTHER:	S. Moshiri	Visiting Fellow	LP, NCI
	E.S. Jaffe	Chief, Hematopathology Section	LP, NCI
	E.W. Chu	Chief, Cytopathology Section	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The cytological diagnosis of malignant lymphoma can be extremely difficult because the cytological features of the malignant cells in small cell and mixed small and large cell lymphomas may be indistinguishable from those of reactive lymphoid cells. We have examined the usefulness of the avidin biotin immunoperoxidase technique and a battery of antibodies to T and B cell markers to the diagnosis of lymphoma in cytological specimens. We conclude that immunocytochemistry is very useful in the cytological diagnosis of non-Hodgkin's lymphoma. Further, it is possible to diagnose the vast majority of lymphomas using only the immunoglobulin light chain markers κ and λ and the T-cell markers Leu-1, Leu 2, and Leu-3.

Project Description

Objectives:

To improve the accuracy of the cytological diagnosis of non-Hodgkin's lymphoma using immunocytochemistry.

Methods Employed:

In addition to routine cytological preparation using standard Millipore filtering techniques and cytospin preparations, aliquots of each specimen are studied using the avidin biotin immunoperoxidase technique and antibodies to B and T cell markers.

Major Findings:

We have investigated 96 specimens, including 66 pleural effusions, 10 ascites, 4 cerebrospinal fluids, and 16 fine needle aspiration specimens. We have found 55 cases to be positive for lymphoma and 41 to be reactive in nature. Of the 55 positive cases, 40 were diagnosed as monoclonal B cell proliferations on the basis of either κ or λ light chain but not both. A diagnosis of T cell lymphoma was made in 7 cases on the basis of aberrant marker phenotype. Of the 8 cases expressing no detectable T cell marker or immunoglobulin, all were positive for T200.

Significance to Biomedical Research and the Program of the Institute:

The cytopathologist can play an important role in staging and diagnosis of recurrences of non-Hodgkin's lymphoma by the examination of fluids and fine needle aspiration biopsies. In most low-grade lymphomas, however, the cytological features of the malignant lymphoid cells are indistinguishable from those of non-neoplastic lymphocytes. We have developed a technique that provides a definitive cytological diagnosis of malignant lymphoma providing a sound basis for staging, evaluation of recurrence, and therapy.

The combination of immunocytochemistry and fine needle aspiration biopsy cytology is a powerful one and given the appropriate cell markers, provides unparalleled opportunity for accurate pathologic diagnosis with the minimum of patient morbidity and expense.

Proposed Course of Research:

We plan to continue our investigation of lymphoid markers on cytological specimens and to extend this approach to the diagnosis of non-lymphoid tumors.

Publications:

Martin, S.E., Zhang, H.-Z., Magyarosy, E., Jaffe, E.S., Hsu, S.-M., and Chu, E.W.: Immunological methods in cytology: Definitive diagnosis of non-Hodgkin's lymphomas using immunological markers for T and B cells. Am. J. Clin. Pathol. 82: 666-673, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09128-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cytologic diagnosis of carcinoma cells in effusions using monoclonal antibodies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S.E. Martin	Surgeon	LP, NCI
OTHER:	S. Moshiri	Visiting Fellow	LP, NCI
	A. Thor	Expert	LTIB, NCI
	V. Vilasi	Visiting Fellow	LTIB, NCI
	E.W. Chu	Chief, Cytopathology Section	LP, NCI
	J. Schlom	Chief, Laboratory of Tumor Immunology and Biology	LTIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

The cytologic diagnosis of metastatic adenocarcinoma in effusions can be very difficult. Not only can malignant cells have very bland cytologic features but reactive mesothelial cells can assume a very atypical appearance. Using the avidin-biotin immunoperoxidase technique on cytospin preparations of pleural and peritoneal effusions, we investigated the reactivity of purified monoclonal antibody B72.3 with benign and malignant effusions. Initial effusion specimens studied were carefully selected to include only cytologically malignant effusions from patients with a history of adenocarcinoma and cytologically benign effusions from patients with no history of adenocarcinoma. Of the 25 malignant effusions studied, 20-90% of the cells demonstrated strongly positive staining with B72.3. There was no detectable staining of cells in reactive effusions. We conclude that immunostaining with B72.3 is useful in the cytologic diagnosis of metastatic adenocarcinoma in effusions.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To determine the efficacy of immunocytochemistry in the cytologic diagnosis of metastatic carcinoma.

Methods Employed:

Millipore filter preparations and Diff-quik stained cytospin preparations of thoracentesis and paracentesis specimens were evaluated on the basis of morphological criteria used. In addition, aliquots of each specimen were studied for immunocytochemical staining using monoclonal antibody B72.3 in the avidin-biotin immunoperoxidase assay. Purified mouse myeloma protein MOPC-21 at equivalent protein concentration was used as a negative control.

Major Findings:

Thirty-five specimens obtained from 29 patients have been studied, including 22 pleural effusions and 13 ascites fluids. Of the 25 cytologically malignant effusions studied for reactivity with the B72.3 antibody, all contained cells which stained strongly and specifically. The percent of malignant cells staining varied between 20 and 90%. In contrast, none of the cells in the reactive effusions stained with the B72.3 antibody.

Significance to Biomedical Research and the Program of the Institute:

While the value of immunoperoxidase to histopathology has been amply demonstrated, the application of immunocytochemistry to cytological specimens is only beginning to be explored. We have developed an assay which takes advantage of the fact that cytologic specimens consist of suspensions of viable cells. Rather than subject the tumor cells to rigorous processing, we make cytospin preparations. This approach offers a number of advantages: (1) it is rapid; (2) it is generally applicable; (3) a small number of cells is sufficient. Using this technology, the purified monoclonal antibody B72.3 has been shown to be sensitive and specific in aiding the cytologic diagnosis of malignancy in cytological effusions.

The combination of this methodology with fine needle aspiration cytology promises to provide a rapid, reliable, and inexpensive approach to the daily diagnosis of cancer.

Proposed Course of Research:

We plan to continue our investigation of monoclonal antibodies useful in the cytologic diagnosis of malignancy and to extend our studies to include fine needle aspiration material.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09129-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Lymph node aspiration in recurrent Hodgkin's disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. Dmitrovsky	Clinical Associate	M, NCI
	S.E. Martin	Surgeon	LP, NCI
OTHER:	A. Krudy	Staff Radiologist	DR, CC
	E.W. Chu	Chief, Cytopathology Section	LP, NCI
	E.S. Jaffe	Chief, Hematopathology Section	LP, NCI
	R.C. Young	Chief, Medicine Branch	M, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.5	.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In this retrospective study covering the 8 year period from 2/76 to 5/84, 19 patients with Hodgkin's disease underwent 64 lymph node aspirations. Of these patients, 17 had suspected Hodgkin's disease with involvement at an inaccessible site. All available cytologic specimens were reviewed to confirm the initial cytologic interpretation. Lymphangiograms were reviewed and lymphangiographic appearance was used as a separate parameter to correlate with cytologic interpretation and the clinical course. This study demonstrated that fine needle aspiration can be useful in diagnosing recurrent Hodgkin's disease, particularly in an inaccessible site, thus avoiding major surgery.

Project Description

Objectives:

To determine the efficacy of fine needle aspiration cytology in the diagnosis of recurrent Hodgkin's disease.

Methods Employed:

Transcutaneous fine needle aspirations were performed by the radiologist using a 22 gauge needle. The prepared smears and syringes were sent to Cytology where smears and Millipore filter preparations were stained by the Papanicolau method. When possible, Diff-quik stained cytospins were made. All cytologic specimens were reviewed by a staff pathologist to confirm the initial cytologic interpretation. Lymphangiograms were reviewed by a staff radiologist.

Major Findings:

Of the 64 aspirations performed on nineteen patients with Hodgkin's disease, aspirations were successful in all but 2 patients. Five of 19 patients had a positive aspirate. Twelve of 19 patients had negative cytology and 2 of 19 had an unsatisfactory aspirate. There were no false positive aspirates for Hodgkin's disease. Of the twelve negative aspirates, 5 were false negatives. In no case did a false negative delay appropriate therapy.

Significance to Biomedical Research and the Program of the Institute:

This study demonstrates that fine needle aspiration can be useful at times in diagnosing recurrent Hodgkin's disease. Further, when inaccessible sites are involved or when concurrent medical problems place patients at risk, major surgery can be avoided by using fine needle aspiration cytology.

Proposed Course of Research:

We plan to continue to evaluate the usefulness of fine needle aspiration cytology in the diagnosis of Hodgkin's disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00520-07 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Surface disposition and fate upon ligand binding of IgE and its receptor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI
OTHER:	C. Isersky	Senior Investigator	A&R, A
	S.J. Mims	Biologist	LP, NCI
	J. Rivera	Biologist	A&R, A

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	2	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Antigenic responsiveness to allergens is imparted to mast cells and basophils by specific membrane binding of allergen binding IgE. Other cells have been shown to bind ligands non-randomly, especially to microvilli (dePetris, Nature 272: 66-68, 1978). Further, cell bound IgE has been shown to survive for prolonged periods of time on the cell surface (Isersky, Rivera, Mims, and Triche, J. Immunol. 122: 1926-1936, 1979). Finally, binding of cell-bound IgE with multi-valent ligand results in rapid internalization without re-expression of both IgE ligand and its receptor. We are studying the native distribution of IgE receptors on the cell surface by two techniques and comparing their fate following ligand binding. Of especial interest is the fate of planar cell surface receptors compared to those on microvilli. In addition, the role of a pre-lysosomal compartment ("CURL") in ligand-IgE-receptor uncoupling and subsequent degradation is being investigated by double label techniques (colloidal gold-ligand and IgE-ferritin or α-receptor ferritin).

Project Description

Objectives:

To delineate the native disposition and subsequent movement and fate of IgE, its ligand and its receptor on basophils, in light of the unique characteristics and function of this receptor and immunoglobulin.

Methods Employed:

1. Radiolabelled IgE binding studies. 2. Electron microscopic autoradiography. 3. IgE-ferritin and colloidal gold - DNP immunoelectron microscopy. 4. Morphometric analysis.

Major Findings:

Detailed analysis indicates no preferential binding to microvilli when values are corrected for actual membrane surface area; uncorrected values suggest binding to microvilli only because of their greater membrane area (4:1). These results with IgE-ferritin were corroborated by studying radiolabelled IgE on basophils detected by EM autoradiography. The latter approach eliminated mass effects of the ferritin label and inadvertent chemical cross-linking of IgE due to the conjugation procedure from consideration.

Significance to Biomedical Research and the Program of the Institute:

Understanding of the mechanism by which individuals retain immediate hypersensitivity to various haptens such as pollen and insect venom for exceedingly prolonged periods of time is hampered by an incomplete understanding of the cellular mechanisms by which IgE is retained on the cells mediating this response. Also, little is known of the fate of IgE, its receptor, or hapten following binding, internalization, and histamine release. This study is designed to clarify the mechanisms involved in these events.

Proposed Course of Research:

Currently, we are studying IgE internalization with colloidal gold-DNP and α-IgE-colloidal gold to study the separate fate of ligand and IgE upon internalization. Subsequently, anti-receptor Ab-ferritin will be studied alone and in parallel with DNP-gold.

Publications:

Furuichi, H., Rivera, J., Triche, T.J., and Isersky, C.: The fate of IgE bound to rat basophilic leukemia cells. IV. Functional association between the receptors for IgE. J. Immunol. 134: 1766-1773, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00545-07 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit an one line between the borders.)*

Extracellular matrix synthesis by human tumors in vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI
OTHER:	A. Modesti	Visiting Fellow	LP, NCI
	S. Scarpa	Visiting Fellow	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The type and amount of matrix proteins synthesized by human tumor cells in vitro appears to parallel that of cultured normal cell counterparts to some extent. We have broadened these observations to a variety of human tumors to determine whether these patterns might allow more precise categorization of the tumor's origins. In addition, we are characterizing a new matrix protein synthesized by some of these tumors. The identity, function, and molecular organization within the extracellular matrix of this component is currently unknown.

GPO 914-218

Project Description

Objectives:

We wish to study extracellular matrix protein (ECM) synthesis by normal and tumor cells in vitro, in cultures established by us from patients with known disease and in lines obtained from commercial sources.

Methods Employed:

These lines will be carefully evaluated for tumor cell growth, using all available techniques (chromosome analysis, density dependent growth, tumor production in nude mice, etc.). Matrix protein synthesis will be evaluated by ion exchange chromatography, slab gel electrophoresis, autoradiography of radiolabelled cells, immunoprecipitation and SDS-PAGE, and electrophoretic transfer with immunodetection.

Major Findings:

1. Eleven Ewing's sarcoma cell lines have been established or investigated and have shown a unique collagen synthetic profile. These cells variably produce types I, III, and IV collagen. This pattern has not been observed in any other tumors or tumor cell lines studied or reported. 2. Five neuroblastoma lines have been studied; three are classic adrenal-origin tumors and synthesize no identifiable stromal collagens (ie, I or III), and are thus distinct from Ewing's sarcoma, which they otherwise resemble. Two of the lines are peripheral in origin, show some morphologic features of Schwann cells, and like those cells, synthesize some stromal collagens as well as FN, LM, and type IV collagen. 3. Preliminary data from osteosarcoma, chondrosarcoma, fibrosarcoma, and carcinoma cultures and, in some cases, tumors, indicated characteristic collagen type synthesis - types I, II, III, and IV, respectively. 4. Laminin and fibronectin have been detected in some tumors but not others, in patterns which reflect ectodermal or mesodermal origin of various childhood tumors.

Significance to Biomedical Research and the Program of the Institute:

Study of in vitro synthesis of matrix components by human tumors will substantiate the applicability of similar studies in animal model systems. These results will 1) have bearing both on our understanding of the origin of these tumors (with obvious therapeutic implications) as well as 2) provide valuable models for the study of the synthesis and assembly of the extracellular matrix by human tissues, as well as providing isolated, purified components of human origin, whose structure and function can then be studied in detail.

Proposed Course of Research:

An appropriate array of tumors has been established in culture (sarcomas, neuroblastomas, Ewing's sarcomas, and lymphomas). Their patterns of matrix protein synthesis have been determined. This data has been presented in abstract form and is submitted for publication. Further work will focus on the effect of

differentiation on ECM synthesis. Also, the character and biological activity of these newly described ECM constituents will be studied.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00874-03 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Neurone-specific enolase in childhood tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI
OTHER:	M. Tsokos	Visiting Scientist	LP, NCI
	R.I. Linnoila	Medical Staff Fellow	LP, NCI
	R. Chandra	Children's Hospital, Washington, D.C.	

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The diagnosis, and thus therapy, of solid tumors of childhood is often difficult due to lack of distinguishing characteristics. This is especially true of Ewing's sarcoma, neuroblastoma, primitive soft tissue sarcomas, and (occasionally) lymphoma. We have evaluated the presence of a specific neural enzyme, neurone-specific enolase (NSE), in paraffin-embedded sections of a diverse group of solid childhood tumors, including previously unrecognized variants of neural tumors, employing immunocytochemistry with antisera to NSE. We find uniform reactivity of all neural tumors with this antibody. No cross-reactivity with non-neural tumors, save a rare example of differentiated rhabdomyosarcoma, was found. We conclude that NSE is a reliable, readily detected marker in even primitive childhood tumors of neural origin. Also, we have defined the neural histogenesis of a newly described, "round cell" tumor of chest wall resembling Ewing's sarcoma, the so-called Askin tumor, which in reality is a form of peripheral neuroepithelioma. Finally, we have recently confirmed the unique character of so-called peripheral neuroepithelioma, which is NSE-positive but which displays hybrid neural and Schwannian morphologic characteristics.

Project Description

Objectives:

To evaluate the use of a neural marker (NSE) in the diagnosis of primitive solid childhood tumor diagnosis.

Methods Employed:

Paraffin sections of various childhood tumors are incubated with monospecific, anti-neurone-specific enolase raised against purified rat brain enolase (γγ isomer), and detected by the Petrusz immunoenzyme bridge technique.

Major Findings:

1. Only tumors of neural crest origin are reactive with the antibody, save for an isolated instance of reactivity with only the differentiated myoblasts of a rhabdomyosarcoma. 2. Normal skeletal muscle, some smooth muscle, nerve, and liver are also reactive. This may reflect cross-reactivity with the α or β isomer subunits, found in liver and muscle, respectively. 3. All neural crest tumors evaluated so far (>40) have been reactive. 4. Previously undescribed or ill-understood neoplasms have been studied and their normal histogenesis confirmed (or refuted). Specifically, the so-called Askin tumor of chest wall has been shown to be a peripheral neuroepithelioma.

Significance to Biomedical Research and the Program of the Institute:

A reliable means of identifying all neural crest tumors in ordinary tissue sections is, to date, unavailable. This technique appears to provide such means. This is critical in determining treatment and assessing results of treatment.

Proposed Course of Research:

This project is now being pursued in conjunction with monoclonal antibody studies of these tumors (See Z01 CB 00899-02 LP).

Publications:

Tsokos, M., Linnoila, R.I., Chandra, R.S., and Triche, T.J.: Neuron-specific enolase in the diagnosis of neuroblastoma of other small, round-cell tumors in children. Hum. Pathol. 15: 575-584, 1984.

Triche, T.J., Tsokos, M., Linnoila, R.I., Marangos, P.J., and Chandra, R.: NSE in neuroblastoma and other round cel tumors of childhood. In Evans, A.E., D'Angio, G.J., and Seeger, R.C.: Advances in Neuroblastoma Research. New York, Alan R. Liss, Inc., 1985, pp. 295-318.

Triche, T.J., Kissane, J.B., and Askin, F.B.: Neuroblastoma, Ewing's sarcoma, and the differential diagnosis of small, round blue cell tumors. In Finegold, M. (Ed.): Pediatric Neoplasia. W.B. Saunders. (in press)

Linnoila, R.I., Tsokos, M., Triche, T.J., Marangos, P.J., and Chandra, R.S.: Evidence for neural origin and PAS positive variants of the malignant small cell tumor of thoracopulmonary region ("Askin tumor"). Am. J. Surg. Pathol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00875-03 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*

Differentiation, matrix proteins, & in vitro invasiveness of human neuroblastoma

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principel Investigetor.) (Neme, title, leboretory, and institute effilietion)*

PI:	M. Tsokos	Visiting Scientist	LP, NCI
OTHER:	S. Scarpa	Visiting Fellow	LP, NCI
	U.P. Thorgeirsson	Visiting Scientist	LP, NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Neuroblastoma is a neoplasm known to show spontaneous histologic maturation in vivo which correlates with a better biologic behavior and prognosis. Extracellular matrix (ECM) proteins, on the other hand, have been shown to influence tumor invasion and metastasis. Production of ECM proteins has been previously reported only for the C1300 murine neuroblastoma cell line. We have studied ECM synthesis (ie, fibronectin (FN), laminin (LM), and collagen type IV) in relation to differentiation of human neuroblastoma in vitro. The qualitative and quantitative differences in ECM protein synthesis by neuroblastomas in vitro before and after differentiation have been assessed by immunofluorescence, polyacrylamide gel electrophoresis and quantitative scanning densitometry of autoradiograms of these gels. Differentiation has been induced by dibutyryl-cyclic AMP and retinoic acid and studied by light and electron microscopy as well as biochemical expression of neurotransmitter enzymes. Finally, the biologic behavior of the neuroblastoma cells before and after differentiation with the above agents was tested in vitro, employing a human amnion invasion assay. Our results indicate that neuroblastoma shows tripartite differentiation in vitro into three main cell types: neuronal, Schwannian and melanocytic. Each cell type has different light and electron microscopic characteristics and exhibits a specific pattern in terms of ECM protein expression. Quantitative studies of the synthesized ECM proteins showed definite changes of all three studied proteins with differentiation. Qualitative and quantitative changes of the neurotransmitter enzymes were also noted. Differentiation was correlated with decreased invasiveness in vitro.

Project Description

Objectives:

To study biologic behavior of neuroblastomas in relation to maturation and extracellular matrix protein synthesis.

Methods Employed:

Immunofluorescence in tissue cultures with antisera to matrix proteins, polyacrylamide gel electrophoresis of biosynthetically radiolabelled matrix proteins, scanning densitometry of autoradiograms and electron microscopy.

Major Findings:

1. Human neuroblastoma produces various ECM proteins in vitro. These include LM, FN, and type IV collagen, but no stromal collagen. 2. Three major cell types emerge in human neuroblastoma after differentiation in vitro: neuronal, Schwannian and melanocytic (tripartite differentiation). Each cell type exhibits a specific pattern of ECM protein expression. 3. Differentiating neuroblastomas seem to produce variable amounts of fibronectin, laminin and type IV collagen and show decreased in vitro invasiveness. 4. A propensity for Schwann cell differentiation is associated with expression of higher levels of fibronectin, laminin and type IV collagen.

Significance to Biomedical Research and the Program of the Institute:

These studies should provide insight into the biological factors involved in neuroblastoma invasion. By experimentally manipulating differentiation, the role of various matrix proteins and their degradation products, as well as cell receptors for each, can be explored.

Proposed Course of Research:

We will focus on the biologic function of laminin and fibronectin as adhesive molecules in specific stages of neuronal, Schwannian, and melanocytic differentiation. The receptor for these moieties will also be studied with regard to distribution, involvement in cell-cell or cell-substrate adhesion, and turnover. The appearance and function of potential cell adhesion molecules with differentiation will be investigated as well.

Publications:

Tsokos, M., Ross, R., and Triche, T.J.: Differentiation of human neuroblastoma into neuronal, Schwannian and melanocytic cells. In Evans, A.E., D'Angio, G.J., and Seeger, R.C.: Advances in Neuroblastoma Research. New York, Alan R. Liss, Inc., 1985, pp. 55-68.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00884-04 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ultrastructural organization of basal lamina

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI
	A. Modesti	Visiting Fellow	LP, NCI
	S. Scarpa	Visiting Fellow	LP, NCI
	T. Kalebic	Visiting Fellow	LP, NCI
	S. Togo	Guest Worker	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The basal lamina has been ultrastructurally characterized as a continuous electron lucent layer (the lamina lucida) adjacent to the cell surface with an overlying electron dense layer (lamina densa), which interfaces with the mesenchymal stroma (collagens and other matrix proteins). Biochemically, the basal lamina is known to contain type IV collagen, laminin, and basement membrane proteoglycan. The actual disposition of these constituents in the l. lucida l. densa, cell surface, and matrix is uncertain, various conflicting ultrastructural studies notwithstanding. Also, the relationship of type V collagen, a so-called cell surface collagen, to the basal lamina, is unknown. We are employing high resolution (ca. 5 nm) immunoelectron microscopy on tissue sections with purified antisera to laminin, type IV collagen, and type V collagen, using appropriate controls, to precisely localize these constituents of the basal lamina and neighboring extracellular matrix.

GPO 914-918

Project Description

Objectives:

To determine the molecular organization of the basal lamina and neighboring extracellular matrix.

Methods Employed:

Human amniotic membrane. 2. Antisera against laminin, type IV collagen, type V collagen, keratin (positive technique control), non-immune serum (negative control), and affinity column eluates (positive and negative controls). 3. Sized protein A-Gold complexes (4 to 12 nm range; ± .8 nm variation in a given preparation), for regular and multiple label experiments. 4. *In vitro* and embedded, sectioned tissue incubations with reagents. 5. Immunoperoxidase EM studies to complement (4), above.

Major Findings:

Initial data indicate that laminin is found at the cell surface and in the proximal l. densa, and to a lesser extent in the l. lucida. Type IV collagen is found in the distal l. densa. Type V collagen appears to be the fine, poorly banded fibrils between the l. densa and the large collagen fibres (types I and III), with possibly some large fibre staining as well.

Significance to Biomedical Research and the Program of the Institute:

Understanding of the biological function of constituents of the extracellular matrix such as laminin, type IV collagen, and type V collagen depends on a knowledge of the molecular organization within tissues. This information is also essential to an understanding of the basic mechanism of cancer cell invasion and metastasis.

Proposed Course of Research:

This project is complete.

Publications:

Modesti, A., Kalebic, T., Scarpa, S., Togo, S., Grotendorst, G., Liotta, L.A., and Triche, T.J.: Type V collagen in human amnion is a 12 nm fibrillar component of the pericellular interstitium. Eur. J. Cell Biol. 35: 246-255, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00899-02 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Small, round cell tumor monoclonal antibody reactivity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, end institute affiliation)*

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI
OTHER:	P. Reynolds	Transplantation Unit, NNMC, USN	
	L. Donner	Pathology Resident, George Washington University Medical Center;	
	Fellow to be named		

COOPERATING UNITS *(if any)*

NNMC Transplantation Unit

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Primitive childhood tumors (ie, classically Ewing's sarcoma, neuroblastoma, lymphoma, and soft tissue sarcoma), are frequently morphologically indistinguishable. Ultrastructural and immunocytochemical techniques are useful but not infallible. Monoclonal antibodies (MoAbs) which recognize neural, lymphoid (HLA-related), and tissue-specific determinants might be useful in distinguishing these entities. We have studied more than 20 cell lines by flow cytofluorometry and more than 40 tumors by frozen section immunocytochemistry with a panel of 12 MoAbs and find reproducible patterns of reactivity which serve to reliably distinguish all neural tumors and hematopoietic malignancies. Ewing's sarcoma is similar to rhabdomyosarcoma, but shows some reactivity with certain neural MoAbs. Peripheral neuroepithelioma is a unique tumor with reactivity intermediate between sarcomas and neural tumors, not unlike Ewing's sarcoma. Thus, most of the tumors are readily recognized, even in the absence of any distinguishing morphologic characteristic. These results have important diagnostic and therapeutic implications, but further study of more tumors is required.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To evaluate an immunologic-based approach to the differential diagnosis of primitive tumors of childhood.

Methods Employed:

1. Tumor cell lines. 2. Frozen sections of same and/or other tumors.
3. A panel of monoclonal antibodies. 4. Fluorescence activated cell sorter.
5. Fluorescence microscope.

Major Findings:

Each of the major categories of childhood tumors can be distinguished from one another by patterns of reactivity with a selected panel of 12 monoclonal antibodies.

Significance to Biomedical Research and the Program of the Institute:

This group of tumors is generally recognized as the most difficult to diagnose, and is often misdiagnosed, despite important differences in treatment and prognosis. This approach provides an alternative, non-morphologic technique for diagnosing tumors.

Proposed Course of Research:

Complement initial feasibility studies (now done) with greater numbers of cases and new MoAbs raised against Ewing's sarcoma. Emphasize frozen sections of excised tumors and correlation with FACS analysis of comparable tumor cell lines.

Publications:

Donner, L., Triche, T.J., Israel, M.A., Seeger, R.C., and Reynolds, C.P.: A panel of monoclonal antibodies which discriminate neuroblastoma from Ewing's sarcoma, rhabdomyosarcoma, neuroepithelioma and hematopoietic malignancies. In Advances in Neuroblastoma Research. New York, Alan R. Liss, Inc., 1985, pp. 161-170.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09125-02 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cytogenetic abnormalities and oncogene expression of small, round cell tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

PI:	M. Israel	Senior Investigator	PB, NCI
OTHER:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI
	C. Thiele	Research Associate	PB, NCI
	J. Whang-Peng	Chief, Cytogenetic Oncology Section	MB, NCI
	E. Gelmann	Senior Investigator	LTCB, NCI
	J. Miser	Expert	PB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

We have encountered a uniform rcp (11:22) translocation in Ewing's sarcoma. This is true of all lines and tumors examined to date (~ 20). It is not true of neuroblastoma, lymphoma, or soft tissue sarcoma. Interestingly, it is also present in a unique childhood tumor, peripheral neuroepithelioma and the closely related chest wall tumor described by Askin et al., the so-called Askin tumor. The break point on chromosome 22 is close to a known oncogene, c-sis. No amplification or rearrangement of c-sis has been detected. In the case of peripheral neuroepithelioma, c-sis is not amplified, but c-myc is. Unlike classic neuroblastoma, N-myc is not expressed. These results serve to emphasize the common abnormality found in Ewing's sarcoma, its distinction from other round cell tumors, and the unique character of peripheral neuroepithelioma.

GPO 914-918

Project Description

Objectives:

1. To detect reproducible chromosomal abnormalities in solid tumors of childhood. 2. To relate oncogene expression to specific tumor types, and potentially to tumor aggressiveness. 3. To detect the relationship, if any, between oncogene amplification, translocation, or rearrangement and *in vivo* or *in vitro* malignancy of a given tumor.

Methods Employed:

1. Cell lines and fresh tissue from patients with Ewing's sarcoma, neuroblastoma, soft tissue sarcoma, or related tumors. 2. Cytogenetics and *in situ* hybridization with various radiolabelled cDNA probes(λ, n-myc, c-myc, c-sis). 3. Southern blots of high molecular weight cell DNA.

Major Findings:

1. All Ewing's sarcoma lines and tumors examined to date possess a reciprocal (11:22) translocation. 2. This translocation of the long arm of 22 occurs close to c-sis, a known oncogene encoding mRNA for platelet derived growth factor. 3. No amplification or rearrangement of c-sis has been detected to date. 4. This abnormality is found in no other solid tumor of childhood, save for peripheral neuroepithelioma.

Significance to Biomedical Research and the Program of the Institute:

Recent results in many laboratories have established the association of oncogene expression with specific tumors. N-myc has been implicated in class II (fatal) neuroblastoma. No such relationship has been detected for other childhood tumors. We are attempting to determine similar relationships between known oncogenes and other tumors. The ability to detect these oncogenes may have important diagnostic, prognostic, and therapeutic implications.

Proposed Course of Research:

More tumors of diverse type will be studied for the presence of specific cytogenetic abnormalities. The expression of certain oncogenes, especially n-myc, c-myc, and c-sis will be assessed by a variety of techniques.

Publications:

Israel, M.A., Thiele, C., Whang-Peng, J., Kao-Shan, C.-S., Triche, T.J., and Miser, J.: Peripheral neuroepithelioma: Genetic analysis of tumor derived cell lines. In Evans, A.E., D'Angio, G.J., and Seeger, R.C.: Advances in Neuroblastoma Research. New York, Alan R. Liss, Inc., 1985, pp. 161-170.

Evans, A.E., Marangos, P., Ladisch, S., Triche, T.J., Zelter, P.M., Hann, H.-W., Donner, L., Moss, T.J., Lampson, L.A., and Voute, P.A.: Discussion: Tumor markers. In Evans, A.E., D'Angio, G.J., and Seeger, R.C.: Advances in Neuroblastoma Research. New York, Alan R. Liss, Inc., 1985, pp. 399-404.

Whang-Peng, J., Triche, T.J., Knutsen, T., Miser, J., Douglass, E.C., and Israel, M.A.: Chromosome translocation in peripheral neuroepithelioma. N. Engl. J. Med. 311: 584-585, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09132-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The role of laminin and its receptor in differentiation of neuroblastoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Tsokos	Visiting Scientist	LP, NCI
OTHER:	P.U. Reddy	Visiting Fellow	LP, NCI
	G.J. Bryant	Expert	LP, NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have recently studied the synthesis and deposition of the extracellular matrix (ECM) proteins laminin (LM), fibronectin (FN) and type IV collagen by human neuroblastoma (NB) cells in vitro, before and after differentiation with dibutyryl-cyclic (dbc) AMP and retinoic acid (RA). The synthesis of all the above ECM proteins was confirmed by polyacrylamide gel electrophoresis (PAGE) of the conditioned culture media of several NB cell lines, after metabolic radiolabelling with 3H-leucine and 3H-proline. Deposition of the proteins on the cell layers was confirmed by immunofluorescence (IF), using antisera against LM, FN and type IV collagen on the cell layers. The pattern of expression of the above proteins by specific morphologic phenotypes suggested to us that these proteins may play a role in neuronal-Schwann cell interaction and hence differentiation of NB. Specifically, the fact that LM was only present on the surface and the cytoplasm of flat cells with Schwannian characteristics and absent from neuronal cells, led us to believe that: (1) cells with features of Schwann cells and neurones were appearing in culture and (2) these cells might recapitulate normal Schwann cell-neurone interaction. In this case, the abundant cell surface LM on Schwann cells might be a chemoattractant for LM-poor, LM-receptor-rich neuronal cells. The existence of such a receptor to laminin has been recently confirmed by several investigators for breast carcinoma, fibrosarcoma and muscle cells (Rao et al., Terranova et al., Lesot et al., and Malinoff and Wicha). Moreover, other authors have shown that neurons extend neurites on laminin substrata. To evaluate the validity of this hypothesis, we will search for the presence of a laminin receptor in NB cell lines and will study its quantitative changes with differentiation and its localization on specific cell phenotypes.

Project Description

Objectives:

To determine the biologic mechanisms of differentiation of NB into neurones and Schwann cells, which may eventually explain similar mechanisms of normal embryonic development of the nervous system as well.

Methods Employed:

Tissue culture, electron microscopy, conjugation of laminin with fluorescein and/or biotin. Radioiodination of laminin with enzymobead reagent. Binding assays with and without competition with cold laminin. Immunofluorescence and immunoperoxidase. Internalization experiments. Attachment experiments on coated substrata.

Major Findings:

(1) NB cells which differentiate predominantly into neuronal cells (CHP-126 cell line with dubutyryl cyclic AMP) attach more readily to laminin-coated substrate than they do to fibronectin or collagen coated ones. The untreated NB cells or these which differentiate predominantly to Schwann cells do not show similar preference to laminin-coated substrata.

(2) Preliminary binding experiments showed the presence of a laminin receptor on the cells of one NB cell line before any treatment with any agents (CHP-126). The receptors were also visualized on the surface of these cells before differentiation by an FITC-laminin ligand.

Significance to Biomedical Research and the Program of the Institute:

Clarification of mechanisms of differentiation of NB cells *in vitro* is important to understand the biologic behavior of this tumor *in vivo* and possibly establish new methods of treatment.

Proposed Course of Research:

These preliminary results support the existence of a laminin receptor in primitive NB cells. A more vigorous proof of their existence by additional experiments and an exact quantitative evaluation before and after differentiation are necessary subsequent experiments to elucidate the role of LM and LM-receptors, if any, in neural development and differentiation.

Publications:

Tsokos, M., Ross, R., and Triche, T.J.: Neuronal, Schwannian and melanocytic differentiation of human neuroblastoma cells *in vitro*. In Evans, A., D'Angio, G.J., and Seeger, R.C. (Eds.): Advances in Neuroblastoma Research. New York, A. R. Liss, Inc., 1985, pp. 55-68.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09133-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Neuroblastoma: The effect of morphologic transformation on metastatic potential

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Tsokos	Visiting Scientist	LP, NCI
OTHER:	S. Scarpa	Visiting Fellow	LP, NCI
	J. Williams	Biologist	LP, NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI
	R. Ross	Dept. of Biol. Sciences, Fordham Univ., Bronx, New York	
	S.J. Mims	Biologist	LP, NCI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

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SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We treated a classic neuroblastoma (NB) and a peripheral neuroepithelioma (PN) with the differentiating agents dibutyryl-cyclic (dbc) AMP and retinoic acid (RA) and evaluated the influence of several factors, such as morphologic transformation, biochemical expression, cell growth, total protein synthesis and extracellular matrix (ECM) protein synthesis to their metastatic capacity in vivo. The latter was evaluated by injecting treated and untreated cells from both lines in the tail vein of 31 nude mice. The mice were sacrificed after the injection and complete autopsies were performed.

The results showed that the same differentiating agents favored a diverse pathway of differentiation in the 2 studied lines. Moreover, Schwannian transformation was associated with a remarkable increase in total protein synthesis and ECM protein synthesis and no changes in cell growth, whereas neuronal transformation was accompanied by a minor increase in total protein synthesis, decrease in ECM protein synthesis and inhibition of cell growth. Schwannian transformation was associated with a higher metastatic potential, when compared to untreated or neuronally transformed cells. The explanation of the latter phenomenon is not known. However, high levels of LM and unsaturated receptors have been alternatively implicated in the high metastatic potential of other cell types and we intend to look for LM and its receptors in the nude mice tumors from metastatic sites.

Project Description

Objectives:

To determine factors associated with a high metastatic potential of NB cells.

Methods Employed:

Tissue culture, cell differentiation *in vitro*, polyacrylamide gel electrophoresis, immunofluorescence, electron microscopy, and tail vein injection of tumors in nude mice.

Major Findings:

(1) Differentiation of classic NB and P.N. into specific cell types *in vitro* (neuronal, Schwannian) is associated with specific biochemical changes, i.e. increase of C.A.T. in neuronal and C.N.P. in Schwannian transformation and increased protein synthesis including increased synthesis and deposition of LM, FN and type IV collagen in Schwannian and decreased in neuronal transformation.

(2) Schwannian transformation is associated with a higher metastatic potential *in vivo*.

Significance to Biomedical Research and the Program of the Institute:

Neuroblastoma is a tumor with variable clinical behavior; factors such as age, stage, and histologic maturation play important roles. Most neuroblastomas are refractory to current treatment. The understanding of mechanisms of metastasis of this tumor is a major goal of pediatric oncology, since it may help identify new treatment modalities. Moreover, histologic evaluation of tumors with more sophisticated techniques, such as detection of LM receptors or LM itself on sections may be of great prognostic value and possibly an indicator of specific treatment in the future.

Proposed Course of Research:

The original results will be confirmed by repeating the experiment with a new batch of nude mice. In addition, the tumors from the mice from both primary and metastatic sites will be frozen and frozen sections will be stained by immunofluorescence using a LM-FITC ligand and anti-LM antibody to evaluate the presence of LM and LM receptors on the cells of primary and metastatic sites. Samples of the tumor will also be evaluated by electron microscopy for detection of subtle morphologic changes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09134-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Solid variant alveolar rhabdomyosarcoma - a rhabdomyosarcoma with poor prognosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Tsokos	Visiting Scientist	LP, NCI
OTHER:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI

COOPERATING UNITS *(if any)*

A. Miser, J. S. Miser, P.A. Pizzo, Pediatric Oncology Branch, NCI; R. Wesley, Biostatistics, NCI; T.J. Kinsella, J. Grayson, E. Glatstein, Radiation Therapy, NCI

LAB/BRANCH

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SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3/4	3/4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Upon reviewing the histologic sections from the primary tumors of 42 patients treated in the rhabdomyosarcoma (RMS) protocol at the NCI, we encountered 15 primitive round cell sarcomas of the soft tissue with no overt pattern, but with cytologic characteristics resembling those of an alveolar RMS. The referring diagnoses on those cases were RMS NOS, embryonal RMS or primitive sarcoma of soft tissues. All these cases clinically behaved in a fashion similar to that of alveolar RMS. For all the above reasons, we termed this primitive subtype of RMS, "solid variant" alveolar RMS.

To evaluate the use of electron microscopy (EM) or immunocytochemistry in distinguishing this primitive RMS from other tumors, we reviewed the electron microscopic (EM) prints on 13 out of 15 cases and employed immunocytochemistry for muscle markers on 14 cases. The markers investigated were skeletal muscle myosin (MYS), myoglobin (NMG), creatine-phosphokinase (CPK) MM and desmin. The results showed that 13 out of 15 cases met the criteria for being diagnosed as RMS. The other 2 should be classified as primitive sarcomas NOS. Seven cases had cells with Z-band material and/or basal lamina and 3 inhibited a fair amount of cytoplasmic filaments. Two to 4 muscle markers were present in all RMS cases. Desmin was the most reliable single marker present in all cases. These immunocytochemical results represent extension of our previous work on RMS and support the myogenous nature of the solid variant alveolar RMS. They also suggest that EM and immunocytochemistry are useful tools to confirm the diagnosis of RMS in doubtful cases.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To define a specific subgroup of RMS associated with a bad prognosis and previously confused with other tumors or embryonal RMS. Also to establish effective methods of proper diagnosis of this entity.

Methods Employed:

Light microscopic evaluation, electron microscopy, immunoperoxidase.

Major Findings:

(1) Primitive RMS is a round cell tumor of soft tissues, with cytologic characteristics identical to those of alveolar RMS and a solid phase of growth, hence the term "solid variant" alveolar RMS.

(2) The majority of these primitive RMS show variable amount of cells with Z-band material in the cytoplasm by EM.

(3) All cases are invariably positive for desmin and 1 or 3 additional muscle markers. The intensity of desmin is higher when Z-band material is present and lower in the absence of Z-band material and presence of intracytoplasmic filaments.

(4) EM and immunocytochemistry are valuable diagnostic tools in primitive RMS.

Significance to Biomedical Research and the Program of the Institute:

The distinction of "solid variant" RMS is crucial in establishing the new NCI classification scheme of RMS. The latter has been proven to be the only histologic scheme which can predict prognosis in childhood RMS according to our study. Since solid variant alveolar RMS was so far confused with an embryonal RMS or other entities (comparison with referring diagnoses), the establishment of firm diagnostic criteria by EM and immunocytochemistry will help the distinction of this entity and hence the establishment of a better treatment. Therefore, distinction of solid variant alveolar RMS is one of the major interests of the pediatric oncology group at the NCI and other institutions.

Proposed Course of Research:

The project is almost completed. A few more cases will be included.

Publications:

Tsokos, M., Howard, R.M., and Costa, J.: Immunohistochemical study of alveolar and embryonal rhabdomyosarcoma. Lab. Invest. 48: 148-155, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09135-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Peripheral neuroepithelioma - A tumor distinct from neuroblastoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Tsokos	Visiting Scientist	LP, NCI
OTHER:	M.A. Israel	Senior Investigator	PB, NCI
	L. Donner	Resident, George Washington University, Washington, D.C.	
	C.P. Reynolds	Transplantation Research Program, National Naval Medical Center, Bethesda, MD	
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Peripheral neuroepithelioma (PN) has been underdiagnosed for many years, because of its histologic resemblance to Ewing's sarcoma in most cases, or occasionally to rhabdomyosarcomas (RMS). Lately, we were able to prove the neuroectodermal nature of the small round cell tumor of thoracopulmonary region ("Askin tumor"), a variant of PN, employing immunoperoxidase to detect the presence of the enzyme neuron-specific enolase (NSE) and electron microscopy (EM). In a wider study of small round cell tumors of childhood, we also found that NSE was only present in neuroectodermal tumors and not in Ewing's sarcoma or lymphoma. However, rhabdomyosarcomas (RMS) were also nonspecifically positive for NSE, and therefore, for distinction of RMS from PN, muscle markers and EM are additional diagnostic tools.

Here we evaluated the histologic, immunocytochemical, cytochemical and EM features of 15 cases of PN and correlated them with clinical presentation. All tumors were extra-adrenal and occurred in diverse soft tissue and bony sites, in post-ganglionic locations. All patients were older than these in classic neuroblastoma (NB) and secreted no catecholamines. All tumors were NSE positive and 50% S-100 protein positive. The EM appearance was overall more primitive but the neural characters were established in most cases. Immunofluorescence with monoclonal antibodies showed a specific pattern of staining for PN, distinct from NB and similar, although not identical to Ewing's sarcoma.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To establish firm morphologic and immunocytochemical criteria for distinction of the entity peripheral neuroepithelial from classic neuroblastoma, Ewing's sarcoma and rhabdomyosarcoma.

Methods Employed:

Light microscopy, electron microscopy, immunoperoxidase and immunofluorescence.

Major Findings:

(1) Peripheral neuroepithelioma, although it may resemble Ewing's sarcoma or rhabdomyosarcoma by light microscopy, has a unique EM appearance in most cases and expresses NSE immunoreactivity, whereas Ewing's sarcoma is negative for NSE and rhabdomyosarcoma and exhibits muscle markers.

(2) Peripheral neuroepithelioma is different from classic neuroblastoma, in that it presents in an older group of patients, in extra-adrenal, postganglionic sites, does not secrete catecholamines, exhibits less morphologic differentiation to neuroblasts, shows more often other neuroectodermal cell populations, such as Schwann cells and melanocytes and exhibits a specific pattern of monoclonal antibodies positive for HLA-class I base determinants (W6/32) and class I associated antigen (BBM.1) and equivocally positive for the neuroblastic antigen HSAN 1.2, in contrast to classic neuroblastoma.

(3) Peripheral neuroepithelioma is intermediate in its reactivity of monoclonal antibodies between classic neuroblastoma and Ewing's sarcoma.

Significance to Biomedical Research and the Program of the Institute:

The clinical course and therapeutic responsiveness of peripheral neuroepithelioma is unknown, since this tumor has been masked so far with other diagnoses, due to absence of firm histologic criteria. The establishment of diagnostic tools for distinction of this entity from other small round cell tumors of childhood has been a necessity for the clinical pediatric oncology group at the NCI, since it will lead to a better understanding of its clinical behavior and prognosis, as well as to institution of the best possible treatment for these tumors. Moreover, prospective distinction of this tumor from Ewing's sarcoma will answer the question if they are indeed different clinical entities, or one is the primitive form of the other.

Proposed Course of Research:

The project is almost completed. Minor additional studies will include detection of choline acetyltransferase and neurofilaments by immunofluorescence, using specific monoclonal antibodies on the 4 peripheral neuroepithelioma cases that we have frozen tissue on, as well as immunostaining of some more Ewing's sarcoma cases with NSE to confirm the specificity of the latter enzyme in the distinction of these two tumors.

Publications:

Linnoila, R.T., Tsokos, M., Triche, T.J., Marangos, P.J., and Chandra, R.S.: Evidence for neural origin and PAS positive variants of the malignant small cell tumor of thoracopulmonary region ("Askin tumor"). Am. J. Surg. Pathol. (in press)

Tsokos, M., Linnoila, R.I., Chandra, R.S., and Triche, T.J.: Neuron-specific enolase in the diagnosis of neuroblastoma and other small, round-cell tumors of childhood. Hum. Pathol. 15: 575-584, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09136-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Classic biologic differences between peripheral neuroblastoma and Ewing's sarcoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Tsokos	Visiting Scientist	LP, NCI
OTHER:	J. Williams	Biologist	LP, NCI
	S.J. Mims	Biologist	LP, NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI

COOPERATING UNITS *(if any)*

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Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This is a new project which was initiated 4 months ago. There is clinical evidence to support that peripheral neuroepithelioma (PN) is different than classic neuroblastoma (NB) and is associated with a much more aggressive clinical behavior than the latter entity. On the other hand, the relationship of PN to Ewing's sarcoma is unknown although there is evidence to suggest that the former may be a more differentiated form of the latter in certain occasions. To evaluate the biologic behavior of these entities on an experimental basis, we will inject tumor cells of well established classic NB (CHP-126, SMS-SAN, IMR-32), PN (TC-32, TC-135, SK-N-MC, and Ewing's sarcoma (TC-71, A4573, 6647) cell lines into the subcutis and tail vein of nude mice and evaluate their tumorigenicity and metastatic potential. The morphologic differentiation of tumors before and after injection into nude mice will be evaluated as well. Preliminary results with only 2 of the cell lines (CHP-126 and TC-32) showed a slightly higher tumorigenic capacity for the PN cell line, when compared to that of classic NB (5/5 mice injected with TC-32 cells had tumor nodules vs. 3 out of 5 injected with CHP-126 cells).

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To determine differences in the biologic behavior of classic NB, PN and Ewing's sarcoma.

Methods Employed:

Tumor cell lines. Injection into nude mice. Electron microscopy. Immunofluorescence and immunoperoxidase.

Major Findings:

The project is fairly new, but there is evidence to suggest that the PN will prove to be a more aggressive biological tumor than classic NB and probably similar to Ewing's sarcoma.

Significance to Biomedical Research and the Program of the Institute:

The biologic behavior of small, round-cell tumors of childhood with superficially similar histology, but different clinical features and/or diverse histogenesis is of great interest among the clinical oncologists at the NCI, who treat these patients under specific protocols.

Proposed Course of Research:

If the initial experiments show remarkable differences, the tumors which will grow in nude mice will be evaluated immunocytochemically for detection of various markers such as choline acetyltransferase, NSE, S-100, LM and LM receptors, as well as for expression of gene products, with various probes and *in situ* hybridization.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09137-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ewing's sarcoma: Differentiation in vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI
OTHER:	A.O. Cavazzana	Guest Researcher	LP, NCI
	S.J. Mims	Biologist	LP, NCI
	J.A. Jefferson	Biologist	LP, NCI

COOPERATING UNITS *(if any)*

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Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2 1/2	1 1/2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The histogenesis of Ewing's sarcoma remains enigmatic, despite much work to elucidate its origins. We have assumed that Ewing's sarcoma, in its usual state of differentiation, lacks any specific features of known childhood tumors. Certain lines of evidence from other studies, such as the presence of a reciprocal (11:22) chromosomal translocation in Ewing's sarcoma and peripheral neuroepithelioma, and similar patterns of reactivity with panels of monoclonal antibodies, have suggested a possible common histogenesis for these otherwise dissimilar tumors. Since neural tumors in general are known to respond to differentiating agents such as dibutyryl cyclic AMP, nerve growth factor, and retinoic acid by developing features of differentiated neural tissues such as neurites and increased numbers of dense core granules, we have treated a series of Ewing's sarcoma tumor cell lines in vitro with these agents under a variety of conditions, alone and in conjunction with one another.

To date, the initial results strongly suggest that at least those tumors which are successfully grown in vitro are intrinsically capable of neural differentiation in response to treatment with these agents. Four of four lines so studied (and reported previously to lack any spontaneous evidence of neural differentiation, even after year of growth in vitro) responded by producing long, slender processes in culture. Ultrastructural examination of these processes revealed dense core granules. Immunocytochemistry with antisera to neuron-specific enolase, an antigen found in neural tissue, was negative prior to treatment but positive afterwards in all four lines. These initial results are being confirmed with other techniques, including catecholamine fluorescence, neurotransmitter enzyme profiles, extracellular matrix synthesis studies, and patterns of monoclonal antibody reactivity.

Project Description

Objectives:

To determine the histogenesis of Ewing's sarcoma and its relationship to other childhood tumors.

Methods Employed:

Tissue culture, electron microscopy, immunocytochemistry with monoclonal and polyclonal antibodies, catecholamine fluorescence, biosynthetic radiolabel incorporation studies of protein synthesis.

Major Findings:

1. Ewing's sarcoma can be induced to develop neural phenotypic characteristics following treatment with appropriate differentiating agents *in vitro*.

2. This neural phenotype closely resembles that expressed by so-called peripheral neuroepithelioma, but not classic childhood neuroblastoma.

Significance to Biomedical Research and the Program of the Institute:

The proper classification and treatment of round cell, primitive tumors, of which Ewing's sarcoma is the most conspicuous example, is entirely empirical to date. Rational treatment of this and related tumors depends on a basic understanding of their inter-relationships and origins. Such information has not been available for these tumors in the past, but is becoming available as a result of these studies.

Proposed Course of Research:

These initial, but very promising, results must be confirmed by more vigorous proofs. Additional studies employing diverse other techniques beyond morphology and immunocytochemistry are underway.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09138-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

In situ hybridization studies of N-myc expression by neuroblastoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. Cohen	Clinical Associate	PB, NCI
OTHER:	R. Seeger	Physician in Chief, Pediatric Hem.-Oncol., Univ. of Southern California	
	M. Israel	Sr. Attending Physician	PB, NCI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI

COOPERATING UNITS *(if any)*

Department of Pediatric Hematology-Oncology, USC, Los Angeles, CA

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2 1/2	2	1/2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Neuroblastoma, alone among extra-CNS childhood tumors, has been shown to express a unique proto-oncogene, N-myc. Further, elevated expression and/or amplification of this oncogene has been correlated with adverse clinical course; no stage I or II patients express the gene abnormally, while over 50% of stage III and IV patients do. Current work indicates these patients fare especially poorly. Nonetheless, no work to date has attempted to correlate the expression of N-myc by individual tumor cells with stage and outcome. Bulk techniques employed to date cannot distinguish a small population of N-myc expressor tumor cells admixed with non-expressors, yet such patients may prove to have a prognosis equally adverse as those with high levels of N-myc expression. This might be the case with the N-myc negative stage III and IV patients reported to date.

The present study will examine N-myc expression as DNA copies and RNA transcripts as detected by in situ hybridization with radiolabelled DNA frgments of the N-myc gene, transcribed in vitro and hybridized to frozen sections of approximately 80 tumors provided by one of us (RS). The incidence of positive tumor cells and their morphology will be assessed in each case, and upon completion of the study, the identity, stage, and status of each patient will be correlated with N-myc expression.

Project Description

Objectives:

To determine whether N-myc expression on a cell-by-cell basis can be correlated with clinical outcome more precisely than existing studies on average N-myc expression by whole extracts of tumor DNA.

Methods Employed:

In situ hybridization of radiolabelled N-myc probe to frozen sections of approximately 80 cases of childhood neuroblastoma obtained from one of us (RS). Autoradiographic detection and morphometric analysis of grains, and correlation with tumor cell morphology, will be employed.

Major Findings:

This study is in its early stages. To date, the approach has been proven technically feasible, and N-myc positive and negative tumors have been successfully distinguished. Study of the patient tumor population noted above is underway.

Significance to Biomedical Research and the Program of the Institute:

Neuroblastoma is a tumor refractory to treatment in most cases, for unknown reasons. The ability to identify such cases prior to treatment, and to understand why they are not amenable to treatment, is a major goal of pediatric oncology, both at the NCI and at every major pediatric oncology program.

Proposed Course of Research:

The 80 tumors noted above, as well as new cases received during the course of the study, will be studied as outlined above. Correlation with morphology of the frozen sectioned tumor, as well as other material available on the case, will be undertaken. Prognostically important variables will be ascertained. Additional studies with other tumors and other oncogenes are anticipated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09139-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Monoclonal antibodies to Ewing's sacoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI
	S. Arakawa	Fogarty Fellow	LP, NCI
	C.P. Reynolds	Tissue Transplantation Unit	NMC, NCR

COOPERATING UNITS *(if any)*

Tissue Transplantation Unit, NMC, NCR

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This is a new project, just commencing. Extensive studies of Ewing's sarcoma and related round cell tumors of childhood have served to distinguish most from one another on the basis of positive reactivity with one or more antibodies. No antibody specific for Ewing's sarcoma, or restricted to a few tumors including Ewing's, has been identified to date. It is the aim of the present study to produce a battery of monoclonal antibodies against selected, well-characterized Ewing's sarcoma lines established in this laboratory. They will then be studied and compared in their reactivity with other monoclonal antibodies previously reported. Any antibody found to be strongly reactive with Ewing's sarcoma cells will be further characterized, with the eventual intention to use same as a potential diagnostic, imaging, and therapeutic tool.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To create an antibody or antibodies which will identify Ewing's sarcoma cells.

Methods Employed:

Tumor cell lines, hybridoma technology, fluorescence activated cell sorter, immunofluorescence.

Major Findings:

None to date. This study commenced June, 1985.

Significance to Biomedical Research and the Program of the Institute:

The lack of a means to positively identify Ewing's sarcoma cells, *in vivo* or *in vitro*, has hampered efforts to precisely define this enigmatic entity. Further, therapeutic strategies such as bone marrow purging have been impossible, due to the lack of a means of identifying tumor cells other than by morphology. Even morphology is nonspecific; no specific feature of Ewing's sarcoma has even been identified. Thus, the availability of a positive identifier of this tumor is much needed for diagnosis and treatment purposes.

Proposed Course of Research:

Cultured tumor cells or specific extracts or fractions will be injected into BALB/C mice, spleens will be harvested, cells fused with myeloma cells, clones established and screened for the production of reactive antibodies, and promising cultures established. Tissue culture supernatants will be concentrated, reacted with cultured tumor cells, and analyzed by cell sorter, in conjunction with pre-existing monoclonal antibodies. Promising antibodies will be tested on frozen sections of banked Ewing's sarcoma tumors and other childhood tumors, and the results analyzed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09140-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

A new high molecular weight extracellular matrix protein

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI
OTHER:	S. Scarpa	Visiting Fellow	LP, NCI
	P.U. Reddy	Visiting Fellow	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8	8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In the course of studying extracellular matrix synthesis by various childhood tumors, we have encountered a previously unidentified, high molecular weight (500,000 D) protein secreted into the conditioned medium of several tumor cell lines. This protein is secreted in connection with laminin, type IV collagen, and fibronectin, but is immunologically distinct therefrom. It is also non-reactive with antibodies to basal lamina proteoglycan. It fails to label with radiolabelled sodium sulfate or glycosyl precursors, which further distinguishes it from conventional proteoglycans. Selective enzymatic degradation studies indicate that the protein is trypsin and pepsin sensitive, but collagenase and GAG degrading enzyme insensitive. Ultrastructural studies of rotary shadowed, purified molecules reveal a single, unbranched chain of over 700 nm length. The molecule co-purifies with laminin and type IV collagen under normal circumstances. Current efforts are aimed at devising preparative purification techniques which will allow purification of quantities sufficient to initiate the generation of monoclonal antibodies and biologic function studies.

GPO 914-918

Project Description

Objectives:

To determine the identity and biologic function of a newly described protein suspected of being an extracellular matrix protein.

Methods Employed:

Tissue culture, biosynthetic radiolabel incorporation, gel permeation and high performance liquid chromatographic purification of conditioned medium proteins, electrophoretic purification, selective degradation studies, monoclonal antibody generation, and tissue immunocytochemistry of normal and neoplastic tissues, using such monoclonal antibodies.

Major Findings:

We have identified a previously undescribed, very high molecular weight (500,000 D), single-stranded (>700 nm) protein which is distinct from all known extracellular matrix proteins, but which is intimately associated with laminin, type IV collagen, and fibronectin in conditioned tissue cultured medium. The function and relationship to known extracellular matrix proteins is under investigation.

Significance to Biomedical Research and the Program of the Institute:

Studies of tumor cell biology, especially the degradation of pericellular extracellular matrix, have focused on the role of discrete elements of the extracellular matrix, such as laminin, in moderating the biologic behavior (manifest as invasion and/or metastasis) of malignant cells. These studies require a detailed and sophisticated knowledge of the structural organization and composition of the extracellular matrix. Such knowledge has only recently begun to accumulate, and is still incomplete. Studies such as this will provide a far more comprehensive picture of how the individual elements of the pericellular matrix are organized, and their function in normal and neoplastic tissues.

Proposed Course of Research:

Current research is hampered by the lack of a reliable means of purifying reasonable quantities of this scarce protein. In the immediate future, such preparative techniques will be perfected. Once successful, our efforts will focus on raising monoclonal antibodies to the protein, and studying its disposition and presumed function, in relationship to other known constituents of the extracellular matrix.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09141-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Clinicopathologic factors in prognosis of rhabdomyosarcoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Tsokos	Visiting Scientist	LP, NCI
OTHER:	B. Webber	Chief, Surgical Pathology, St. Jude Children's Research Hospital, Memphis, TN	
	D. Parham	Surgical Pathology, St. Jude Children's Research Hospital, Memphis, TN	
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI

COOPERATING UNITS *(if any)*

Pediatric Oncology Branch and Radiation Therapy Branch, NCI; Pediatric Oncology and Radiation Therapy Branch, St. Jude Children's Research Hospital

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

159 cases of rhabdomyosarcoma (RMS), 42 from the NCI and 117 from St. Jude Children's Research Hospital treated similarly and staged according to the Intergroup Rhabdomyosarcoma Study (IRS) criteria were reviewed by 4 pathologists and subclassified according to 3 histologic schemes, i.e. the classical scheme (embryonal, alveolar, pleomorphic), Palmer's scheme (monomorphous round cell, anaplastic, other) and the NCI scheme ("bad prognosis RMS, including the alveolar and solid variant alveolar RMS and good prognosis RMS, including all subcategories of embryonal RMS). Survival curves by histology, age, site grouping and stage were constructed according to the Kaplan and Meier method and compared for homogeneity and a pre-specified trend with the simple and stratified Mantel-Haenszel test. Finally, a multivariate Cox regression analysis was employed for each one of the 3 histologic schemes to evaluate the independent influence of histology on survival and the possible superiority of one scheme over the other two.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

This project is a combined effort among the pathologists, pediatric oncologists, and radiotherapists of the NCI to establish factors that influence the prognosis of childhood RMS.

Methods Employed:

Histologic examination, statistical methods, i.e. Kaplan and Meier, Mantel-Haenszel and multivariate Cox regression analysis.

Major Findings:

1. Histologic classification is an important independent prognostic factor in childhood RMS, only when the NCI classification scheme is employed ($p = 0.005$). The other two classification schemes show no statistically significant independent influence of histology in prognosis (p values 0.10 and 0.194).

2. In addition to overt alveolar RMS, another group of RMS, the solid variant alveolar was recognized. The latter shows similar cytologic characteristics with the previous one but no overt alveolar pattern.

3. Age above 15 years old is associated with an ominous prognosis and no histologic scheme has statistically significant predictive value in children over 15 years of age.

4. Stages III and IV RMS are associated with a poor prognosis compared to I and II. All three histologic schemes have statistically significant predictive value only in stage III RMS. Given that stage III RMS is characterized by grossly residual disease, one should probably consider a more aggressive local treatment (complete surgical excision) for all stage III RMS with aggressive histology.

5. Site groupings into good and bad sites influenced prognosis. The NCI classification scheme had predictive value in good sites ($p = 0.002$) and to a lesser degree in bad sites (0.09). None of the other schemes had prognostic value by site grouping.

Significance to Biomedical Research and the Program of the Institute:

A new histologic scheme with significant predictive value for prognosis, independent from other factors, is instituted for the first time in childhood RMS. This new scheme identifies the alveolar RMS and its solid counterpart as bad prognosis RMS and suggests the need for a more aggressive treatment of this category of RMS. The fact that the scheme works the best on stage III tumors gives the hint for a more aggressive local treatment for the RMS with bad histology vs. the good histology RMS. The results may help institute the best treatment for childhood RMS. At the moment, there are findings to believe that embryonal RMS should be treated conservatively, whereas alveolar RMS should be treated surgically with additional chemotherapy and radiation

therapy. The treatment of childhood RMS is of major interest among the pediatric oncologists at the NCI, since childhood RMS is one of their principal projects.

Proposed Course of Research:

The project is finished. Experimental models will be introduced by infection of cells from embryonal and alveolar RMS in nude mice to test the clinical findings on an experimental basis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09142-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biologic behavior of alveolar and embryonal rhabdomyosarcoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Kouraklis	Visiting Fellow	LP, NCI
OTHER:	M. Tsokos	Visiting Scientist	LP, NCI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have recently concluded from a clinicopathologic study on childhood rhabdomyosarcoma (RMS) that there is a great biologic difference between the embryonal and alveolar histologic subtypes of RMS. To confirm this observation on an experimental basis, we will inject the subcutis and tail vein of nude mice with cells of both subtypes of RMS, from well-established cell lines and will observe their tumorigenicity and metastatic potential. The resulting tumors will be processed for paraffin and EM blocks and for frozen section analysis and in situ hybridization.

GPO 914-918

Project Description

Objectives:

To determine differences in the biologic behavior of embryonal and alveolar RMS.

Methods Employed:

Nude mice injection with tumor cells; electron microscopy; immunofluorescence; immunoperoxidase.

Major Findings:

The project has not been initiated yet. Dr. Kouraklis is expected to arrive in August.

Significance to Biomedical Research and the Program of the Institute:

The knowledge of differences in the biologic behavior of embryonal and alveolar RMS is obligatory in establishing the best therapeutic schemes and evaluating prognosis in this group of tumors. Oncologists at the Pediatric Oncology Branch, NCI, are currently treating patients with RMS under a major protocol, to answer questions as to the best treatment for the disease.

Proposed Course of Research:

If the experiments show remarkable difference in the behavior of the two histologic subtypes of RMS, we will obtain a model to study differences in terms of gene expression products and/or extracellular matrix synthesis which may be implicated in the different biologic behavior of the tumor. Therefore, both cell lines and mice tumors will be studied for the expression of the gene of the laminin receptors, vs. other oncogenes (N-myc, ras, etc.). The synthesis of various types of collagens and laminin will also be studied in the available cell lines.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09143-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Differentiation of human retinoblastoma

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Tsokos	Visiting Scientist	LP, NCI
OTHER:	A.P. Kyritsis	Visiting Associate	LVR, EI
	G.J. Chader	Chief, Laboratory of Vision Research	LVR, EI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP, NCI

COOPERATING UNITS *(if any)*

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Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Monolayer cultures of the Y-79 human retinoblastoma cell line were obtained recently by growing the cells on substrata specifically covered with poly-O-lysine and fibronectin. The cells thus attached were studied by light and electron microscopy, immunofluorescence and immunoperoxidase before and after differentiation with various agents, such as dibutyryl-cyclic AMP (dbc-AMP) and sodium butyrate (Nabut). Serum-supplemented and serum-free media were used. Mere attachment of the cells resulted in minor degrees of morphologic differentiation, which was enhanced after the addition of the agents. Round cells with processes, as well as flat, substrate-adherent cells appeared. Specific neuronal and glial markers were expressed by the cells in various conditions. Moreover, ultrastructural characteristics of neuronal, photoreceptor, glial and pigmented cells were observed. The results support differentiation of primitive retinoblastoma cells, such as those of the Y-79 line, into various cell types of normal human retina, i.e. photoreceptor, neuronal, Müller and pigmented epithelial cells. These findings not only support origin of retinoblastoma from a primitive cell of the optic disc, but also offer a system to study cell interactions and tumor cell responses to various therapeutic agents.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To investigate the histogenesis of human retinoblastoma, and to establish a system of cell interaction, which will help to understand developmental mechanisms of human retina.

Methods Employed:

Tissue culture differentiation experiments, immunofluorescence, immunoperoxidase, electron microscopy.

Major Findings:

1. Retinoblastoma cells can attach on substrates covered with poly-D-lysine and fibronectin.

2. Retinoblastoma cells differentiate into photoreceptors, neuronal, glial and pigmented epithelial cells. The pathway of differentiation can be controlled by specific agents.

3. Neuronal and/or glial cell markers are expressed by specific cell types, after differentiation. The neuronal or photoreceptor cell markers detected in the cells include choline-acetyl transferase (C.A.T.), neuron-specific enolase and interphotoreceptor protein whereas the glial markers include glial fibrillary acidic protein and myelin basic protein.

4. By electron microscopy, several specific morphologic characteristics were seen: a) rosette formation with microvillous processes towards the lumen and occasional centrioles (photoreceptor cell differentiation); b) long cell processes with neurosecretory granules (neuronal differentiation); c) cells with darker cytoplasm, intermediate filaments and well-developed intercellular attachments (glial cell differentiation); d) cells with melanosomes (pigmented epithelial cells).

Significance to Biomedical Research and the Program of the Institute:

The differentiation of retinoblastoma appears to be analogous to neuroblastoma. Because both tumors are neuroectodermal and have many other features in common, the findings in these tumors complement each other and help establish a unifying concept of differentiation and biologic behavior of neuroectodermal tumors in general. New methods of treatment may be the result of such studies.

Proposed Course of Research:

Mechanisms of interactions among the emerging cell types will be studied. As an immediate step, we will study the expression of laminin receptor by the retinoblastoma cells and find if we can control the expression of this receptor manipulating the conditions of the culture to induce specific types of differentiation.

Publications:

Kyritsis, A.P., Tsokos, M., and Chader, G.J.: Attachment culture of human retinoblastoma cells: Long-term culture conditions and effects of cyclic AMP. Exp. Eye Res. 38: 411-421, 1984.

Kyritsis, A.P., Tsokos, M., Triche, T.J., and Chader, G.J.: Retinoblastoma: origin from a primitive neuroectodermal cell? Nature 307: 471-473, 1984.

Tsokos, M. and Kyritsis, A.P.: Neuroblastoma, retinoblastoma, medulloblastoma: Primitive neuroectodermal tumors with common characteristics. A review. Mat. Med. Greca (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00523-06 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Complex carbohydrate released from mammalian cells by trifluoroacetolysis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.A. Zopf	Chief, Biochemical Pathology Section	LP, NCI
OTHER:	G.C. Hansson	Visiting Fellow	LP, NCI
	J. Cashel	Biologist	LP, NCI
	K. Nakahara	Biologist	LP, NCI
	S.J. O'Brien	Chief, Section on Genetics	LVC, NCI

COOPERATING UNITS *(if any)*

T.R. Chen, Research Scientist, ATTC, Rockville, MD

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Carbohydrate chains released by trifluoroacetolysis of whole tissues, tissue fractions, or cells grown in culture, are easily recovered in nearly quantitative yield and reconstituted to their native form. Analysis of the majority of oligosaccharides containing six or fewer monosaccharide units is performed by combined gas chromatography and mass spectrometry of permethylated, N-trifluoroacetylated oligosaccharide derivatives. Analysis for certain specific oligosaccharides is carried out by radioimmunoassay using antibodies produced against purified oligosaccharides coupled to polypeptide carriers. It is anticipated that the repertoire of oligosaccharide chains produced by cells or tissues will reflect states of cellular differentiation and reveal potential cell surface markers.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To separate and identify the major oligosaccharide chains present in mammalian cells and to correlate the occurrence of specific oligosaccharide structures with states of cellular differentiation.

Methods Employed:

The human colorectal carcinoma cell line SW1116 expresses an antigenic ganglioside (the 19-9 antigen) not detectable in normal intestinal mucosa. Previously we used GC/MS analysis to establish the following novel structure for the ganglioside:

NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-ceramide
4
|
Fucα1

To determine the final steps in the pathway of biosynthesis of this ganglioside, we have incubated microsomal enzyme preparations with two oligosaccharide acceptors:

NeuAcα2-3Galβ1-3GalNAcβ1-3Galβ1-4Glc

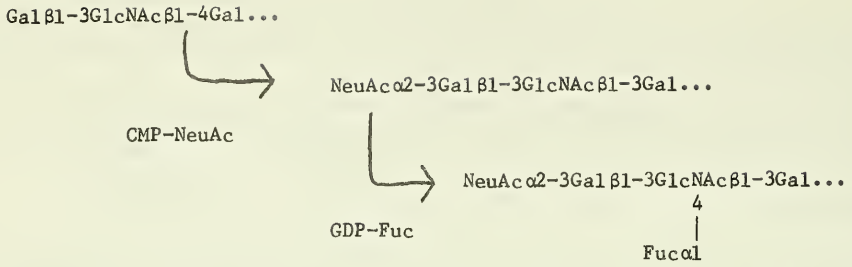
and

Galβ1-3GlcNAcβ1-3Galβ1-4Glc
4
|
Fucα1

and with [^{14}C]-labeled GDP-fucose or CMP-NeuAc. Delipidated and desalted mixtures of oligosaccharide products are analyzed using a newly-developed method for affinity chromatography of oligosaccharides on monoclonal antibodies bound to protein A-Sepharose. Parallel analyses are made using thin-layer and paper chromatography. To determine the chromosomal locations of the gene that code for the glycosyl transferase that adds fucose to form the Lewis a blood group antigen (LNFII above), we have used Sendai virus to fuse mouse DAP cells with SW1116 and cloned the mouse/human hybrids to obtain cell lines with stable patterns of chromosomal segregation. Cell lines are screened for the Lewis antigens by *in vitro* metabolic labeling with [^{14}C]-fucose and thin layer chromotography of purified neutral glycolipids.

Major Findings:

Biosynthesis of the sialyl-Le^a antigen proceeds by addition of NeuAc to a type I blood group chain precursor followed by addition of fucose to the sialylated type I chain as shown below:



These findings explain the observation that individuals of the Le(a-b+) blood group do not express the sialyl-Le^a antigen in normal or tumor tissues. They also suggest that the immediate precursor to the sialyl-Le^a antigen, NeuAcα2-3Galβ1-3GlcNAcβ1-3Gal..., probably occurs in increased amounts in Le(a-b-) individuals in the same normal and malignant tissues where sialyl-Le^a is found in Le(a+b-) and Le(a-b+) individuals. Thus the precursor of sialyl-Le^a might serve as an oncofetal marker for colorectal cancer in Le(a-b-) patients.

More than 10 mouse-human cell lines that express the Lewis antigens have been cloned. Four lines that fail to express the Lewis antigens also are established. Chromosomal analysis of these cell lines is in progress.

Significance to Biomedical Research and the Program of the Institute:

The colorectal carcinoma antigen 19-9 (sialyl-Le^a) was first detected in attempts to produce a tumor-specific monoclonal antibody. A diagnostic kit useful in follow-up for tumor recurrence is now commercially marketed by Centocor Corporation. It would be of interest to understand the biochemical regulation of this antigen and its relationship to oncogenesis. As a first step, we are determining chromosomal localization of the genes that code for the glycosyl transferases responsible for biosynthesis of the sialyl-Le^a structure.

Proposed Course of Research:

Enzymatic studies will continue using purified LSTa-ceramide ganglioside and LNFII-ceramide neutral glycolipid as acceptors. Additional cell lines lacking Lewis antigens will be selected to provide sufficient material for chromosomal assignment of the Lewis glycosyl transferase. Glycolipids of these cells will be subsequently analyzed by GC/MS after trifluoroacetolysis.

Publications:

Hansson, G.C. and Zopf, D.A.: Biosynthesis of the cancer-associated sialyl-Le^a antigen. J. Biol. Chem. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00525-06 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of oligosaccharides by combined gas chromatography-mass spectrometry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.A. Zopf	Chief, Biochemical Pathology Section	LP, NCI
OTHER:	J. Cashel	Biologist	LP, NCI
	E.A. Kabat	Consultant	IRP, NIADDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Separation of reduced and permethylated oligosaccharides by gas chromatography can be facilitated by the use of a fused silica capillary column coated with methyl silicon. The presence of N-acetylhexosamines in oligosaccharides increases their retention time and interferes with efficient GC separation. Transamidation of hexosamines by trifluoroacetolysis followed by reduction, removal of O-trifluoroacetyl groups and permethylation, dramatically reduces the retention time of hexosamine-containing oligosaccharides and permits separation of oligosaccharides containing up to six monosaccharide units, regardless of how many of these are hexosamines. The mass spectra of permethylated oligosaccharides with N-trifluoroacetylated amino sugars show unexpectedly high abundances of mass ions containing the N-trifluoroacetyl group. As many of these ions are large, they provide useful information regarding oligosaccharide structure.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To develop methods for separation and analysis of oligosaccharides by gas chromatography and mass spectrometry.

Methods Employed:

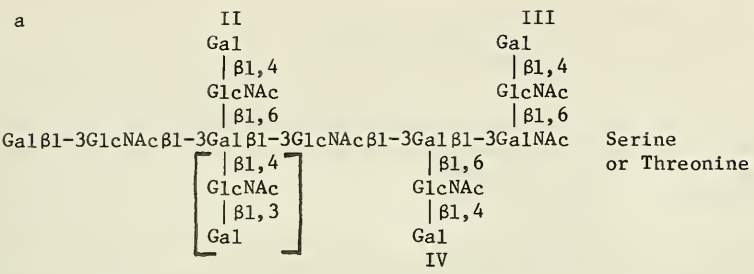
Following trifluoroacetolysis, oligosaccharides are treated with sodium borohydride and methanolic ammonia and finally are permethylated. N-trifluoroacetylated and permethylated oligosaccharide alditols are separated by gas chromatography using a fused silica capillary column coated with methyl silicon. The column effluent is passed without separation into a mass spectrometer.

Major Findings:

Analysis of oligosaccharides as permethylated, N-trifluoroacetylated alditols can be accomplished by combined gas chromatography-mass spectrometry for molecules containing up to seven monosaccharide units including two hexosamines. Standards prepared by trifluoroacetolysis of purified glycolipids and glycoproteins with known carbohydrate structures enable identification of compounds according to retention time on GC under standard conditions and mass spectra. Oligosaccharides from the core regions of blood group substances, proteoglycans, and glycoproteins have been analyzed. For example, the following oligosaccharides were separated and identified in mixtures from HPLC or paper chromatography:

Galβ1-3GalNAc-ol[3]
GlcNAcβ1-6GalNAc-ol
Galβ1-3GlcNAcβ1-6(3-deoxy)GalNAc-ol
Galβ1-3GlcNAcβ1-6GalNAc-ol
Galβ1-4GlcNAcβ1-6GalNAc-ol
GlcNAcβ1-3Galβ1-3GalNAc-ol
Galβ1-3 [GlcNAcβ1-6] GalNAc-ol
Galβ1-3 [Galβ1-4GlcNAcβ1-6] GalNAc-ol
Galβ1-3GlcNAcβ1-3Galβ1-3GalNAc-ol
GlcNAcβ1-3Galβ1-4GlcNAcβ1-6GalNAc-ol
GlcNAcβ1-3Galβ1-3 [Galβ1-4GlcNAcβ1-6] GalNAc-ol
Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ1-3Galβ1-3GalNAc-ol
Galβ1-3GlcNAcβ1-3 [Galβ1-4GlcNAcβ1-6] Galβ1-3GalNAc-ol
Galβ1-3GlcNAcβ1-3Galβ1-3 [Galβ1-4GlcNAcβ1-6] GalNAc-ol

From these structures and information from Dr. Kabat's previous studies, the following composite was prepared, incorporating all of the known structural elements in the core oligosaccharides of human blood group active mucins:



Significance to Biomedical Research and the Program of the Institute:

Structural analysis of oligosaccharides released from biological glycoconjugates usually requires purification and multiple analytical procedures to establish sugar sequence, linkage positions, and anomeric configuration. The gas chromatography/mass spectrometry method under development permits a direct estimate of structural diversity in oligosaccharide mixtures and, in many cases, identification of oligosaccharides according to retention time and mass spectrum by comparison with standards. This approach has enabled structural analysis of compounds present in mixtures that cannot be resolved by any known alternate method. Complex carbohydrates are constituents of many biologically active molecules and play a role in many biochemical recognition events. Rapid structural analysis of these molecules is vital to obtaining an understanding of their role in biological processes. Many recently published studies suggest that mucins produced by human tumors may express carbohydrate antigenic structures lacking on mucins of normal tissue. Factors that regulate biosynthesis of the complete carbohydrate chains of mucins can be determined only when the structures of precursor chains and methods for their routine analysis are known.

Proposed Course of Research:

Studies have been carried out on more than thirty standard oligosaccharides derived from human milk, glycolipids, asparagine-linked chains of glycoproteins, and other sources. Additional oligosaccharides derived from human blood group substances, proteoglycans, and urine are under study. The information derived from studies of the mucin core of soluble human blood group substances will be used as a basis for comparing oligosaccharides from tumor mucins with those of normal mucins from the same individual.

Publications:

Nilsson, B. and Zopf, D.A.: Oligosaccharides released from glycolipids can be analyzed by gas chromatography - mass spectrometry. Arch. Biochem. Biophys. 222: 628-648, 1983.

Wu, A.M., Kabat, E.A., Nilsson, B., Zopf, D.A., Gruezo, F.G., and Liao, J.: Immunochemical studies on blood groups. LXXI. Purification and characterization of radioactive [^{3}H] reduced di- to hexa-saccharides produced by alkaline β-elimination-borohydride [^{3}H] reduction of Smith degraded blood group A active glycoproteins. J. Biol. Chem. 259: 7178-7186, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00556-03 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Expression of glycolipids in lymphocyte subpopulations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.A. Zopf	Chief, Biochemical Pathology Section	LP, NCI
OTHER:	K. Schroer	Senior Assistant Surgeon	LP, NCI
	M. Ugorski	Visiting Fellow	LP, NCI
	K. Wasniowska	Visiting Fellow	LP, NCI
	J. Phung	Biologist	LP, NCI
	J. Cashel	Biologist	LP, NCI
	J. Fernandez	Biological Laboratory Technician	LP, NCI

COOPERATING UNITS *(if any)*

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Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Neutral glycolipids are differentially expressed in functionally distinct subpopulations of murine lymphocytes. Subpopulations of B cells can be studied by examining hybridoma lines derived from fusion of splenic B lymphocytes with the mouse myeloma SP2/0. We are analyzing total neutral glycolipids from hybridomas by thin layer chromatography and by GC/MS analysis of oligosaccharides after trifluoroacetolysis. Hybridomas from Balb/c splenocytes express glycolipids containing from two to five simple sugars. These include globoside and its precursors as well as asialo-GM2 and 2' fucosyllactosyl ceramide. The goal of this project is to correlate expression of oligosaccharide chains of glycolipids with functional parameters of B cell subsets such as responsiveness to Type I and Type II antigens.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To examine the major glycolipids of hybridomas derived under conditions selective for early and late maturing B cell subsets.

Methods Employed:

A mouse myeloma (SP2/0) that expresses only mono- and dihexosylceramides is fused to mouse splenocytes. The fusion products express neutral glycolipids of both the myeloma and lymphocyte parent cells. Thus, glycolipids larger than dihexosyl ceramide are contributed by the B cell parent lymphocyte. Hybridomas (10^7 cells) are labeled in vitro for 48 h with [^{14}C]Gal and [^{14}C]GlcNAc and purified total neutral glycolipids are separated by thin layer chromatography and detected by autoradiography. Selected clones are expanded to 10^9 cells and the purified total neutral glycolipids are subjected to trifluoroacetolysis to release the oligosaccharide chains. The oligosaccharide mixture is reduced, permethylated, and analyzed by GC/MS.

Major Findings:

The composition of neutral glycolipids produced by cloned hybridomas derived from normal, healthy, immunologically "unstimulated" mice varies widely: a few hybridoma clones produce only lactosylceramides, whereas others produce various mixtures of higher glycolipids including globotriaosyl- and globotetraosylceramides, gangliotriaosyl- and gangliotetraosylceramides, 2'fucosyllactosylceramide (2'FLcer), and some higher fucosylated glycolipids whose oligosaccharide structures are not yet fully characterized. In contrast, a panel of IgM-secreting hybridomas prepared from splenocytes of LPS-treated Balb/cJ mice all produce identical glycolipids that include lactosyl ceramide, 2'FLcer, and globotriaosylceramide. A panel of anti-dextran secreting hybridomas from mice immunized with the polysaccharide antigen Dextran 1355S all produce identical neutral glycolipids that include the same structures seen in hybridomas from LPS-treated animals plus globoside. Anti-TNP secreting hybridomas from splenocytes of Balb/cJ mice immunized with TNP-KLH display a characteristic uniform pattern of glycolipids that includes a large amount of 2'FLcer.

Since 2'FLcer had not been described in lymphoid cells prior to these observations, we explored its biosynthesis and possible occurrence in mouse B and T cells, plasma cells, lymphomas, and plasmacytomas. Very small amounts of 2'FL, not detected in our initial studies, were observed in the myeloma cell line SP2/0 and in the precursor cell lines from which it was derived, P3X8 and MOPC 21. Other Balb/c myelomas also contain 2'FLcer, some in large amounts. In contrast, only trace amounts of 2'FLcer appear in B lymphocytes purified by panning Balb/c splenocytes on anti-immunoglobulin coated petri plates. Analysis of 2'FLcer in Balb/c plasma cells is in progress.

Studies on the biosynthesis of 2'FLcer indicate that the fucosyltransferase that catalyses the reaction:

$$\text{Gal}\beta1\text{-4Glc-cer} \xrightarrow[\text{GDP-Fuc}]{} \text{Fuc}\alpha1\text{-2Gal}\beta1\text{-4Glc-cer}$$

is identical to the enzyme that catalyses biosynthesis of higher H-blood group active glycolipids containing terminal Fucα1-2Galβ1-4GlcNAc... sequences. Addition of fucose to Galβ1-4Glc-cer in vitro proceeds much more slowly than addition to lacto- lactoneo-, and gangliotetraosylceramides. Thus, the presence of large amounts of 2'FLcer in some hybridomas and plasmacytomas suggests that extremely high levels of Fucα1-2 to Gal fucosyltransferase exist in some subsets of late B cells and plasma cells. Some glycoproteins of these cells may likewise be fucosylated by this enzyme.

Significance to Biomedical Research and the Program of the Institute:

The complex interactions between cells of the immune system are mediated by numerous recognition events at cell surfaces. We are studying the major glycolipids expressed by lymphocyte subpopulations in order to determine whether any correlations can be made between the carbohydrate chains displayed on the cell surface and the functional state of the lymphocyte. Results thus far strongly suggest that subpopulations responsive to different classes of antigens express different glycolipids. If additional studies confirm these correlations, then specific glycolipids can be used as markers for stages of cellular differentiation and their possible roles in immunoregulatory pathways should be explored. Immunoregulation of anticarbohydrate responses will be critically important for understanding immune surveillance against tumors since many recent attempts to define oncofetal antigens by hybridoma antibodies have yielded antibodies that react with cell surface carbohydrates.

Proposed Course of Research:

Comparison of hybridomas from newborn, adult, and xid mice will be pursued. Hybridomas made against Type I and Type II antigens early and late in development and in defective and non-defective reciprocal crosses will be studied. Splenic lymphocytes will be selectively purified using hybridoma antibodies against specific glycolipids and their responsiveness to different classes of antigens will be determined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00879-02 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Nucleotide sequencing of hybridoma antibodies of Vh-GAC family

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, leboratory, and institute affiliation)*

PI:	K.R. Schroer	Senior Surgeon	LP, NCI
OTHER:	J. Phung	Biologist	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mouse immune response to streptococcal group A carbohydrate (GAC) utilizes many different gene segments from a diverse array of precursor Vh genes. The gene family number is estimated at 10 and the diversity of response in normal mice at over 200 different antibodies. The response is clonally restricted in each mouse immunized, but each mouse appears to express a different clonally homogeneous antibody in its serum in spite of genetically common background. The common denominator of similarity in these antibodies is their usage of highly homologous Vh gene segments, and identical Dh, and Jh segments. The serum response to GAC is entirely deficient in CBA/N mice which makes this an ideal system in which to investigate possible constraints on gene segment utilization.

Recent evidence indicates that up to 5% of CBA/N B cells utilize the Vh-GAC gene segment(s) in their Ig rearrangements: thus, 5-10% of plasma cells are stainable by an anti-Vh^{gac} antisera, and 5% of serum antibodies are positive for this serologic Vh marker by radioimmunoassay. This high frequency of expression of Vh gene(s) is greater than has previously been reported with any other Vh specific antisera, and provides an ideal experimental system to probe the Vh repertoire of CBA/N mice by examination of their B-cell derived hybridomas for structural constraints on combinatorial use. It is likely that a Vh-gene with such a high rate of representation in mouse B cells would probably serve redundantly as the V region for many antibodies of different binding specificity when combined with different D, and J segments along with different L chains. Thus, the family of inulin (B-2--1 fructosan) binding proteins share the Vh-GAC V-gene but use different joining segments and L chains. This V-gene has only been observed in anti-carbohydrate antibodies of normal mice and its paradoxical high expression by CBA/N B cells which fail to respond to carbohydrate antigens remains unexplained.

Project Description

Objectives:

To compare the structural correlates of hybridoma antibodies with their antigenic specificities and serologically defined families.

Methods Employed:

Hybridoma derived cell lines making antibodies bearing V_H determinants of the GAC-J606 family derived from Balb/c and CBA/N mice will be used to obtain deduced sequences of heavy and light chains of their antibodies. The sequencing method is that of Sanger et al. using oligonucleotide primed cDNA synthesis on an mRNA template to generate nucleotide sequence of V_H, D_H, J_H and of V_K, J_K from which the amino acid sequence is obtained.

Major Findings:

An antibody isolated from a Balb/c mouse bears the V_H^{GAC} V region, but differs from the usual inulin/GAC antibodies in specificity, and combinatorial use of D_H, J_H and light chain genes. This demonstrates that a single V_H gene may participate in antibodies of varying antigenic specificity. Four antibodies of CBA/N origin are currently being prepared for sequence analysis.

Significance to Biomedical Research and the Program of the Institute:

Primary structural analysis of antibodies is the definitive method for classification of families of antibodies. Such basic scientific efforts enhance our understanding of the organization and expression of the immune repertoire of antibodies.

Proposed Course of Research:

To sequence members of the above mentioned families of antibodies and compare this new information with previously established sequence data.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00887-02 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunochemical studies of cell surface glycoproteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.A. Zopf	Chief, Biochemical Pathology Section	LP, NCI
OTHER:	K.R. Schroer	Senior Surgeon	LP, NCI
	K. Wasniowska	Visiting Fellow	LP, NCI
	C.M. Reichert	Chief, Autopsy Service	LP, NCI
	M.H. McGinniss	Research Biologist	BB, CC

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Membrane glycoproteins behave as either carbohydrate or peptide antigens and occasionally express antigens that combine specific structural elements from both sugar and peptide moieties. Immune responsiveness to cell surface glycoproteins has not been studied systematically. We are characterizing the fine specificities of autoantibodies against glycoproteins of human erythrocytes from patients with altered immunologic states. In addition, we are preparing hybridomas that secrete monoclonal antibodies against various portions of the carbohydrate and peptide moieties of human glycophorin A, the major sialoglycoprotein of human erythrocytes.

GPO 914-916

Project Description

Objectives:

To examine immunogenicity and immunochemical properties of glycoproteins associated with cell membranes.

Methods Employed:

Hemagglutinating human antisera with serological properties suggesting specificity for glycoprotein surface antigens are studied by hemagglutination inhibition using purified membrane glycoproteins and chemically defined glycopeptides derived from the glycoproteins. Hybridoma antibodies from animals immunized with whole cells are screened for hemagglutinating activity and for binding to purified membrane glycoproteins by ELISA.

Major Findings:

1) An auto-agglutinin from serum of a patient with nucleoside phosphorylase deficiency is inhibited by purified glycophorin A and by glycopeptides that include amino acids 40-61. These results agree with serological tests that assign the antibody En^aFS specificity.

2) Several monoclonal antibodies that agglutinate human red blood cells and bind purified glycophorin A have been prepared. One of these is specific for the blood group N located at the amino terminal end of N-glycophorin A and glycophorin B. Others react with regions of the glycophorin A molecule closer to the membrane bilayer.

Significance to Biomedical Research and the Program of the Institute:

Immune recognition of cell surface glycoproteins may play a major role in surveillance and destruction of tumor cells. Antibodies with specificities for epitopes that can be precisely localized with respect to the surface membrane can provide basic information concerning steric requirements for antibody recognition of surface determinants. The same antibodies can be used to explore the tissue distribution and ontogeny of cell surface components and aberrant expression of glycoproteins as tumor markers in malignant tissue.

Proposed Course of Research:

We will continue to characterize the fine specificities of antibodies against human erythrocytes and initiate studies of the tissue distribution of specific epitopes of glycophorin A.

Publications:

McGinniss, M.H., Wasniowska, K., Zopf, D.A., Straus, S.E., and Reichert, C.M.: An erythrocyte Pr auto-antibody with sialoglycoprotein specificity in a patient with purine nucleoside phosphorylase deficiency. Transfusion 25: 131-136, 1985.

Wasniowska, K., Reichert, C.M., McGinniss, M.H., Schroer, K.R., and Zopf, D.A.: Two monoclonal antibodies highly specific for the blood group N determinant. Glycoconjugate Journal (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00559-03 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell matrix receptors role in metastases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI
OTHER:	U. Wewer	Visiting Fellow	LP, NCI
	C.N. Rao	Visiting Fellow	LP, NCI

COOPERATING UNITS *(if any)*

Laboratory of Developmental Biology and Anomalies, NIDR

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1.5	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Laminin, a glycoprotein of basement membranes, binds to a specific receptor on the surface of neoplastic and non-neoplastic cells. Laminin exhibits saturatable and competible binding to the surface of cultured living cells, or to isolated plasma membranes from cells or tissue. The binding coefficient is 2 nM with 50,000 receptors per cell. The receptor was isolated from murine and human carcinomas and melanomas. It has a molecular weight of approximately 67,000 daltons. The laminin receptor purified from human breast carcinoma plasma membranes was used as an antigen to generate monoclonal antibodies (mAbs). Using immunoblotting, the mAbs recognize a single ≈ 67,000 dalton protein among all the proteins extracted from breast carcinoma plasma membranes. The mAbs differed in their ability to block binding of laminin to the plasma membrane receptor. Antibody LR1 inhibited virtually 100% of the specific binding of laminin to both the isolated human breast - carcinoma plasma membranes or the living MCF-7 cells. In contrast, antibody LR2 had no effect on laminin binding under identical conditions. Thus, the two types of mAbs may recognize different functional domains on the laminin receptor. Immunoperoxidase staining of human epithelium or cells newly attaching to the basement membrane indicates that the laminin receptors are polarized at the base of the cell.

GPO 914-918

Project Description

Objectives:

a) To isolate and chemically characterize the laminin receptor; b) to produce polyclonal and monoclonal antibodies to the laminin receptor; c) to study the genetics and regulation of the receptor; d) to identify receptors for other matrix components; and e) to study the biosynthesis and regulation of the receptor.

Methods Employed:

Plasma membranes were isolated from cells in log phase of growth or from human breast carcinoma biopsy samples. The plasma membrane homogenate was solubilized in 0.1% Triton X 100, 1.0 - 2.0 mg protein/ml. After centrifugation at 30,000 g for 45 min, the supernate was collected and incubated with SM2 Biobeads (Bio-Rad) to remove the Triton. Iodination of the laminin ligand and the plasma membrane extract was performed using the lactoperoxidase method. Laminin receptors were measured on living cells in suspension. After EDTA removal, the cells were incubated in complete media under constant agitation at 37° for 2 hr. The labeled ligand plus 250 fold excess unlabeled ligand was added and the incubation was continued for 2 hr at 25°C. The cell-bound and free ligand was separated by centrifugation. Binding assays on plasma membrane extracts were performed using one member of the ligand or receptor pair bound to solid phase nitrocellulose Millipore SCWP circles or cyanogen bromide activated Sepharose 4B. In the latter case, 25 µg of laminin or plasma membrane extract protein bound to cyanogen bromide activated Sepharose 4B (100 µl) was mixed with an equal amount of 25 mM Tris, 5 mM $MgCl_2$ and $CaCl_2$, pH 7.4 and 100 µl of this buffer containing 0.1% BSA. ^{125}I-labeled plasma membrane extract (10^8 cpm/mg) or ^{125}I-labeled laminin (10^9 cpm/mg) was added in a total volume of 100 µl, diluted with the buffer. Laminin affinity chromatography was performed using purified laminin cross linked to Sepharose 4B. ^{125}I-labeled plasma membrane extract was incubated 15 hr in the laminin-Sepharose affinity column (1 x 15 cm) at 4°C. The unbound radioactivity was washed with 40 ml of 25 mM Tris, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.9% NaCl, pH 7.4. The bound activity was eluted with 0.2 M glycine HCl, acetic acid, SDS, or urea, pH 3.5, immediately neutralized with 1.0 M Tris saline, and lyophilized. The proteins were identified by slab gel electrophoresis and autoradiography. The number of laminin receptor sites and the Kd were calculated by Scatchard analysis. The laminin receptor isolated from human breast carcinoma tissue was used as an antigen for preparing monoclonal antibodies. Thirteen hybridomas (cloned twice) were obtained which produced IgM antibodies to the receptor as judged by solid phase RIA and immunoblotting.

Major Findings:

Human breast carcinoma isolated plasma membranes exhibit saturatable binding to ^{125}I-laminin at 25°C. Specific binding is a high proportion of total binding. Based on displacement of bound labeled laminin by unlabeled free laminin, the binding coefficient was estimated to be approximately 2 nM. MCF-7 breast carcinoma cells (clone 5A9) demonstrated saturatable binding to ^{125}I-laminin. Specific binding was 80 to 90% of total binding. Scatchard analysis is linear

with a Kd of 1.8 nM and approximately 80,000 receptors per cell. Monoclonal antibodies (mAbs) were produced against the purified human breast carcinoma laminin receptor. The antigen was isolated from human breast carcinoma tissue plasma membranes as described previously. The purified antigen was verified to retain its high affinity for laminin. Two antibodies (LR1 and LR2) which differ in their effects on laminin binding to the receptor were identified. By solid phase radioimmunoassay screening, mAb LR1 and LR2 bound with a relatively equal titer to the purified receptor. Control mAbs (B6.2 and B1.1) which recognize breast carcinoma cell surface antigens distinct from the laminin receptor failed to react with the purified antigen. As expected, the antilaminin receptor mAbs failed to bind to the purified laminin ligand itself. Using immunoblotting, both LR1 and LR2 recognized a single 67,000 dalton component among all the proteins extracted from the plasma membranes of human breast carcinoma tissue. The monoclonal antibodies also bound with equal titer to suspended plasma membranes obtained from human breast carcinoma tissue.

LR1 and LR2 exhibited markedly different effects on the binding of laminin to isolated plasma membranes or cells. When added together with the labeled ligand mAb LR1 produced a dose dependent inhibition of specific laminin binding. In contrast, mAb LR2 had no effect. Control mAb (B6.2) directed against a different breast cancer antigen abundant on MCF-7 cells also had no effect on laminin binding. The laminin receptor was found to be polarized at the base of attaching cells or whole epithelium. However, it was disorganized in distribution in tumor tissue sections.

Using the murine melanoma model, cell surface bound whole laminin stimulated metastases, whereas the C_1 receptor binding fragment of laminin markedly inhibited metastases. For a constant amount of laminin or the C_1 fragment, the effect was greater when fewer cells were injected. This is in keeping with the existence of a limited number of receptors and less ligand available per cell as the cell number increases. Separate metastases assays were performed in which a constant amount of cells were treated with different concentrations of ligand.

Significance to Biomedical Research and the Program of the Institute:

a) A pharmacologic agent which blocks the laminin receptor could be clinically useful in treatment of metastatic disease; b) measurement of laminin receptors in biopsy specimens of human breast cancer could be of diagnostic usefulness.

Proposed Course of Research:

a) To correlate laminin receptor content with disease stage; b) to sequence the receptor binding domain on laminin; c) prepare synthetic peptides which block the receptor binding domain on laminin; and d) to plan clinical trials to study the distribution and tissue localization of labeled receptor binding fragments.

Publications:

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Roberts, D.D., Rao, C.N., Magnani, J.L., Spitalnik, S.L., Liotta, L.A., and Ginsburg, V.: Laminin binds specifically to sulfated glycolipids. Proc. Natl. Acad. Sci. USA 82: 1306-1310, 1985.

Rao, C.N., Margulies, I.M.K., and Liotta, L.A.: Binding domain for laminin on type IV collagen. Biochem. Biophys. Res. Commun. 128: 45-52, 1985.

Horan Hand, P., Thor, A., Rao, C.N., Schlom, J., and Liotta, L.A.: Expression of laminin receptor in normal and carcinomatous human tissues as defined by a monoclonal antibody. Cancer Res. (in press)

Thorgeirsson, U.P., Turpeenniemi-Hujanen, T., and Liotta, L.A.: Mechanisms of tumor invasion and their potential therapeutic modifications. In Mihich, E. (Ed.): Biological Responses in Cancer, Vol. 4. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00888-02 LP

PERIOD COVERED

The genetic mechanism and gene expression in the metastatic process

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

October 1, 1984 to September 30, 1985

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	U.P. Thorgeirsson	Visiting Scientist	LP, NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI
	T. Turpeenniemi-Hujanen	Visiting Fellow	LP, NCI
	M.E. Sobel	Senior Investigator	LP, NCI
	J.E. Talmadge	Chief, Preclinical Screening Lab.	FCRF, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our previous studies on the genetic mechanism of metastases have shown that the metastatic phenotype can be conferred upon benign cells through somatic cell hybridization, as well as through DNA transfection. NIH-3T3 cells transfected with either DNA from malignant cells or the isolated Ha-ras oncogene are metastatic in NIH nude mice, but NIH-3T3 cells transfected with normal embryonic DNA or spontaneously transformed in vivo are not metastatic. To further evaluate the role of activated ras genes in metastases, the DNA levels and expression of the ras specific sequences were measured in both NIH-3T3 transfectants and metastatic NMU-induced rat mammary carcinoma containing mutationally activated c-Ha-ras oncogene. Experimental and spontaneous metastases from 3T3/T24 DNA and 3T3/c-Ha-ras transfectants demonstrated a variability in the levels of the Ha-ras specific DNA sequences which were either lower or higher than the primary tumor. Similarly, a variability was noticed in the levels of Ha-ras specific DNA and RNA sequences in ten lung metastases derived from a primary nitroso-methylurea (NMU)-induced rat mammary carcinoma. The DNA levels ranged from 10% to 300% of that measured in the primary tumor. Three out of 10 metastases expressed very low RNA levels (5-10% of the primary) of the Ha-ras oncogene. Less variability was detected in NMU tumors after long-term passage in vivo, both within each group of primary tumors and the metastases, as well as between the groups of primaries and metastases. Similarly, minor differences were seen in the Ha-ras levels between metastatic and nonmetastatic primary NMU tumors. This work shows that activated ras genes transfected into NIH-3T3 cells can produce metastases in NIH nude mice. Although the ras genes can set in motion the metastatic cascade, their heterogeneous expression in metastases suggests that they do not play a major role in the growth of metastases.

Project Description

Objectives:

1. To study the role of activated ras oncogenes in metastases using a) NIH-3T3 transfectants and b) rat mammary carcinoma induced by nitroso-methylurea (NMU).

2. To study protein patterns of metastatic tumors using the NMU-induced rat mammary carcinoma as a model. Quantitative and qualitative differences in both cellular and secreted proteins will be looked for.

Methods Employed:

In vivo metastases assays using tail vein injections for experimental metastases and subcutaneous injections of tumor cells for spontaneous metastases. A dissecting microscope is used for enumeration of lung metastases. Cell lines are established from primary and metastatic tumor explants in Primaria culture flasks.

Extraction of genomic DNA and total RNA from cell cultures and tumors. Southern blot analysis on tumor cell DNA digested with different restriction endonucleases. Northern blot analysis on tumor cell RNA, and quantitation of ras specific DNA and RNA sequences by slot blot analysis. Nick translation and ^{32}P-labeling of different ras oncogene probes used for hybridization of DNA and RNA blots. Identification of p21 protein present in NMU induced tumors and normal mammary glands by gel electrophoresis and immunoblotting of cell lysates.

Collection of cellular and secreted tumor cell proteins for two-dimensional gel electrophoresis and computerized analysis. The following types of breast tissues are analyzed: normal, lactating, metastatic carcinoma and nonmetastatic carcinoma. The tissue is homogenized and the plasma membranes then separated from the cytosol fractions. The secreted tumor proteins are collected in vivo into special chambers placed in close contact with the subcutaneously transplanted tumor.

Major Findings:

Metastases were produced in NIH nude mice after intravenous or subcutaneous injection of NIH-3T3 transfectants containing activated ras oncogenes with or without human tumor DNA sequences. Tumorigenic, spontaneously transformed NIH-3T3 cells and nontumorigenic NIH-3T3 cells transfected with normal embryonic DNA failed to metastasize. NIH-3T3 cells transfected with the v-H-ras oncogene or DNA from either acute lymphocytic or myelogenous leukemia that contained an activated N-ras oncogene produced lung metastases after tail vein injection but did not produce spontaneous metastases from a subcutaneous tumor cell inoculation. The more malignant NIH-3T3 transfectants containing genomic DNA from T24 human bladder carcinoma or the T24-Ha-ras oncogene alone produced both experimental and spontaneous metastases during the 4-6 week period of observation in NIH nude mice. Variability in the levels of ras specific DNA sequences were noticed in both experimental and spontaneous metastases; some were lower and others were higher than the ras levels of the parent cell line.

NMU-induced rat mammary carcinomas that possess mutationally activated Ha-ras oncogene were used as an experimental model to study ras expression in metastases. NMU-induced tumors rarely metastasize. A metastatic NMU-induced mammary carcinoma was found in a Sprague Dawley rat that was sacrificed 9 months after a single IV injection of NMU (5 mg/100 g body wt.). The primary tumor that weighed 76 g had metastasized extensively to the lungs. The microscopic appearance of the primary tumor was that of a well-differentiated infiltrating duct carcinoma and the 10 individual lung metastases that were isolated revealed mixed pattern of well-formed glands and sheets of poorly differentiated cells. Tumor tissue from the rat primary and the 10 individual metastases was expanded subcutaneously in NIH nude mice for DNA and RNA isolation. The levels of the Ha-ras specific DNA sequences in the 10 metastases derived from a single primary tumor were variable. Some were lower, others higher than the primary tumor, ranging from 10% to 300% of the primary. Similarly, a variable expression of the Ha-ras was detected in RNA extracted from the metastases. RNA from three of the metastases expressed very low levels of the Ha-ras or 5-10% of the primary, while others expressed higher levels, up to 300%, of the primary tumor. Cell lines that were derived from the primary tumor and each of 5 metastases were injected intravenously into NIH nude mice. No significant differences in the metastatic capacity between the primary and the metastases were detected.

Three metastatic NMU-induced tumors (NMU_1, NMU_2, NMU_3) were studied that had been passaged in vivo in syngeneic rats for 3 months (NMU_3), 1 year (NMU_2) and 5 years (NMU_1). Minimal variability in the Ha-ras specific DNA levels has been detected in the passaged NMU tumors measured so far.

To further evaluate whether the Ha-ras may determine the metastatic behavior through oncogene amplification, we compared 8 primary metastatic with 6 nonmetastatic NMU tumors. No difference in the Ha-ras specific levels was detected between the metastatic and the nonmetastatic NMU tumors.

Significance to Biomedical Research and the Program of the Institute:

Since most cancer deaths are related to metastatic spread, it is of utmost importance to understand the genetic and biochemical determinants for the metastatic phenotype. Studies on clones from human and animal tumors have demonstrated metastatic diversity within the same tumor, and an ever-changing nature of the heterogeneous tumor cell populations. Therefore, it is often difficult to correlate different biochemical properties of tumor cells with their metastatic behavior. In the past, it has not been solved whether the etiology of metastases is of epigenetic or genetic nature; or both. Our approach to use DNA mediated gene transfer has revealed that the metastatic phenotype can be conferred upon NIH-3T3 cells through transfection with DNA from malignant cells or activated ras oncogenes. Furthermore, the NMU-induced rat mammary carcinoma model that includes multiple primary metastatic and nonmetastatic tumors as well as metastases represents a powerful tool to identify both genetic and biochemical differences. Work is now in progress involving computer analysis of two-dimensional gels on cellular and secreted proteins from normal rat breasts, primary breast carcinoma and metastases. Any qualitative or quantitative differences between the proteins of normal, nonmetastatic and metastatic cells may give a lead to possible genetic aberrations occurring in the metastatic cells.

Proposed Course of Research:

1. Further comparative studies on spontaneously transformed NIH-3T3 cells that are tumorigenic but nonmetastatic, and ras transfected NIH-3T3 cells that are both tumorigenic and metastatic in NIH nude mice. The studies will involve genetic and biochemical approaches to detect how the activated ras oncogenes alter the NIH-3T3 phenotype to make it metastatic.

2. The NMU-induced rat mammary carcinoma model will be used to detect both genetic and epigenetic differences in metastatic and nonmetastatic primary tumors as well as in metastases. Tissues from numerous primary NMU tumors and individual metastases are available for study. Computerized analysis will be performed on cellular and secreted tumor proteins separated by two-dimensional gel electrophoresis. Tumors will be homogenized and divided into membrane-associated and cytosol proteins. The secreted tumor proteins will be collected in vivo into specific chambers closely attached to the subcutaneously transplanted NMU tumors. Special emphasis will be placed on type IV collagenase, which is a basement membrane degrading enzyme secreted by tumor cells during tumor invasion.

3. Further studies will be undertaken on the activated Ha-ras oncogene in NMU-induced rat mammary carcinoma. Ha-ras oligonucleotide probe will be synthesized containing the point mutation that codes for the glycine-glutamic acid substitution in the 12 codon. This oligonucleotide probe will only hybridize with the activated Ha-ras and will be used to verify that all the primary NMU tumors and the metastatic deposits possess the activated Ha-ras oncogene. Breast tissues from normal, lactating and pregnant rats have been collected, and will serve as negative controls for the oligonucleotide probe. They will also be used for quantitation of the Ha-ras oncogene at different stages of normal activity as compared with the malignant breast tissue.

The p21 protein levels of the NMU tumors will be measured by immunoprecipitation and immunoblotting of the tumor lysates. For comparison, the p21 protein will also be identified in ^{35}S labeled cell lines that are being established from the various NMU tumors. Finally, karyotypic analysis will be made on NMU tumor cell lines and evaluated in relation to other genetic and epigenetic parameters under study.

Publications:

Thorgeirsson, U.P., Turpeenniemi-Hujanen, T., Williams, J.E., Westin, E.H., Heilman, C.A., Talmadge, J.E., and Liotta, L.A.: NIH-3T3 cells transfected with human tumor DNA containing activated ras oncogenes express the metastatic phenotype in nude mice. Mol. Cell. Biol. 5: 259-262, 1985.

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Thorgeirsson, U.P., Turpeenniemi-Hujanen, T., and Liotta, L.A.: Mechanisms of tumor invasion and their potential therapeutic modifications. In Mihich, E. (Ed.): Biological Responses in Cancer, Vol. 4. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00889-02 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chrerecters or less. Title must fit on one line between the borders.)*

Laminin receptor: Biochemical characterization

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Neme, title, laboratory, and institute affiliation)*

PI:	U. Wewer	Visiting Fellow	LP, NCI
OTHER:	C.N. Rao	Visiting Associate	LP, NCI
	I.M.K. Margulies	Biologist	LP, NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A cell surface receptor for the extracellular matrix glycoprotein laminin has been demonstrated via several methods in our laboratory. The proposed study is designed to biochemically characterize the receptor for laminin with regard to its biochemical properties in the membrane and behavior after ligand binding. Large quantities of purified receptor have been prepared and submitted for protein sequencing.

Project Description

Objectives:

1. Prepare large quantities of homogeneously pure human laminin receptor.
2. Obtain the entire protein sequence of the receptor.
3. Determine the sequence of the laminin binding region of the receptor.
4. Biochemically characterize the receptor itself and the fate of the receptor ligand complex.

Methods Employed:

The receptor is isolated from plasma membrane preparations of human carcinoma or placenta tissue. The receptor is purified using detergent extraction of the cell membranes, laminin affinity chromatography and standard protein chemistry techniques. Antibodies are prepared in rabbits using the purified antigen. Protein sequencing is performed using a micro-sequemat.

Major Findings:

The laminin receptor is purified from human carcinoma and placenta using laminin affinity chromatography. The receptor is eluted using acetic acid, followed by SDS. The receptor is a single chain of 67,000 m_rw after reduction and a size of $\approx$ 55,000 m_rw before reduction. No cross reactivity with serum components is observed using monoclonal antibodies to the laminin receptor. An ELISA assay was developed for the receptor. The ELISA was used to assay the elution fractions. Approximately 50 µg of purified receptor have been prepared. This material is being prepared for protein sequencing. Polyclonal antibodies to the human laminin receptor have been prepared and these are being characterized. Defined protease-derived fragments of the receptor have been prepared.

Significance to Biomedical Research and the Program of the Institute:

A true appreciation of the laminin receptor transmembrane location, homology with other cellular proteins, and relationship to oncogene products, will be derived from the protein sequence of the receptor. This work can lead to new pharmacologic strategies to block the receptor or measure it in human cancer tissue.

Proposed Course of Research:

1. Complete the amino acid sequence of the entire laminin receptor.
2. Compare the sequence with known proteins using a data base.
3. Prepare a synthetic protein which mimics or blocks the laminin binding on the receptor.

Publications:

Liotta, L.A., Horan Hand, P., Rao, C.N., Bryant, G., Barsky, S.H., and Schlom, J.: Monoclonal antibodies to the human laminin receptor recognize distinct structural sites. Exp. Cell Res. 156: 117-126, 1985.

Liotta, L.A., Wewer, U.M., Rao, C.N., and Bryant, G.: Laminin receptor. (Book Chapter) Neuroscience. (in press)

Wewer, U.M., Albrechtsen, R., Rao, C.N., and Liotta, L.A.: The role of basement membranes in malignancy. (Book Chapter) In Kühn, K. (Ed.): Rheumatology - An Annual Review. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00890-02 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Laminin receptor in leukocytes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G.J. Bryant	Expert	LP, NCI
OTHER:	E. Schiffmann	Research Biochemist	LP, NCI
	C.N. Rao	Visiting Associate	LP, NCI

COOPERATING UNITS *(if any)*

S.E. Martin and E. Magyarosy, Cytopathology Section, LP, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	.2	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous studies on the mechanism of leukocyte traversal of basement membranes showed that rabbit peritoneal exudate cells (PMN) preferentially used laminin, a major constituent of basement membrane, to attach to another component, type IV collagen. PMN also responded chemotactically to nanomolar levels of laminin. We have now determined that PMN possess a receptor for laminin. Scatchard analysis using ^{125}I-laminin indicate a single class of saturable high affinity binding sites (kd = 6.15 nM/L) on PMN and 3.6 x 10^4 sites per cell. A chymotryptically derived fragment of laminin, C_1, which retains the cell binding site gave similar results. Immunoperoxidase studies using monoclonal antibodies to the laminin receptor indicated the presence of the receptor on PMN cell surfaces. PMN responded chemotactically to nanomolar levels of C_1 and laminin, a result consonant with binding data. PMN chemotaxis to a formyl peptide was markedly inhibited by the monoclonal antibodies, suggesting that the laminin receptor may be required for PMN chemotaxis in general. Our results suggest that PMN extravasation across basement membranes is aided both by reversible attachment of the cells to laminin in the matrix and by chemotaxis to a gradient of soluble intact and possibly degraded laminin. These characteristics have much in common with those of highly metastatic tumor cells.

Project Description

Objectives:

To examine the laminin receptor on neutrophils (PMN) and to determine the role of this receptor in functions of the neutrophil.

Methods Employed:

Assay of binding of ^{125}I-laminin and ^{125}I C_1 laminin fragment to neutrophils from rabbit peritoneal exudates was performed in accordance with procedures developed with the use of tumor cells. The effects of monoclonal antibodies to the laminin receptor upon neutrophil chemotaxis was measured with the aid of the modified Boyden chamber filter apparatus.

Major Findings:

Rabbit neutrophils were found to bind ^{125}I laminin with high affinity. Binding of the enzymatically derived fragment C_1 yield a similar high affinity and an estimated 5.0×10^4 sites per cell. Immunoperoxidase studies using monoclonal antibodies to the laminin receptor demonstrated the receptor binding site on the surface of PMNs. These antibodies inhibited the chemotactic response of neutrophils to the leukoattractant F Met-Leu-Phe.

Significance to Biomedical Research and the Program of the Institute:

Neutrophils must traverse endothelial basement membrane to reach extravascular foci of infection. Such migration is probably initiated by attractants emitted by bacteria. The cells then attach to the basement membrane prior to penetration. Since basement membrane contains both laminin and type IV collagen as major constituents, both laminin receptors and laminin itself on the neutrophil surface would aid these cells in traversing the basement membrane. Therefore, neutrophils may behave in a very similar fashion to that of metastatic tumor cells in crossing biological barriers to a target site. It is conceivable that different but highly motile cell types may use the same molecular anchoring mechanisms in migrating across basement membranes.

Proposed Course of Research:

Research on this project has been completed. We have presented the preliminary results in abstract at the FASEB meeting in April, 1985. The completed study is being submitted for publication in the Journal of Leukocyte Biology.

Publications:

Liotta, L.A., Horan-Hand, P., Rao, C.N., Bryant, G., Barsky, S.H., and Schlom, J.: Monoclonal antibodies to the human laminin receptor recognize structurally distinct sites. Exp. Cell Res. 156: 117-126, 1985.

Terranova, V.P., DiFlorio, R., Vasanthakumar, G., Schiffmann, E., Liotta, L.A., Thorgeirsson, U.P., Siegle, G.P., and Gallin, J.I. Laminin promotes rabbit neutrophil motility and attachment. J. Clin. Invest. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00891-02 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemotaxis in tumor cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

PI:	E. Schiffmann	Research Chemist	LP, NCI
OTHER:	R. Mandler	Graduate Student	LP, NCI

COOPERATING UNITS *(if any)*

LDBA/NIDR

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are studying biochemical events characteristic of malignant tumor cells, which must be highly motile while invading tissue and metastasizing to distant sites. We have found that a number of metastatic cell lines produce and respond to autocrine motility factors. A partially purified material from the conditioned media of a human melanoma cell line was found to be a protein with an Mr of about ~ 55 KD. The material induces a strong chemotactic response in the producer cells and appears to exert its action by perturbing membrane phospholipid metabolism of the cell. We have extended these observations to 3T3 cells and their transformed metastatic counterparts. We find that the transformed cells produce and respond well to autocrine factors but poorly to platelet-derived growth factor (PDGF). The nontransformed cells, on the other hand, respond well to PDGF but to a lesser extent to the autocrine factors of the transformed cells. The nontransformed cells do not produce autocrine motility factors. These results suggest that an important characteristic of the metastatic phenotype of malignant cells is their ability to produce and respond to autocrine motility factors.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To study the biochemical aspects of chemotactic motility in tumor cells and to determine whether such motility is correlated with the metastatic potential of various tumor cells.

Methods Employed:

The modified Boyden chamber filter assay for chemotaxis was used. Both high performance liquid chromatography and ordinary gel filtration procedures were employed to isolate active material from conditioned media.

Major Findings:

A material that is strongly chemotactic for the human melanoma cell line A2058 has been partially purified from the serum-free conditioned media of these cells. The activity is sensitive to protease digestion and heating at 100° for 5 minutes, a finding indicative of its protein nature. The activity is heterogeneous, as inferred from the results of high performance liquid chromatography (HPLC) and an Mr ~ 55 KD has been estimated from gel electrophoresis results with the active fractions from HPLC. The motility induced by partially purified factor is both chemokinetic and chemotactic, as determined from the 'checkerboard' matrix assay. Agents which inhibit methylation of membrane phospholipids, such as deaza-adenosine, and inhibitors of phospholipase A2, such as quinacrine, markedly reduce the motility response without toxicity to the cells. The attractant itself produces a sustained methylation of phospholipids. These results suggest that perturbation of the metabolism of membrane phospholipids may be involved in transducing the chemotactic signal to a motile response in the cell. In addition, low levels of pertussis toxin protein (10^{-8}M) markedly inhibit cell motility, an observation suggesting a role for G proteins in the chemotactic response.

It has also been found that 3T3 cells transfected with each of three *ras* oncogenes and possessing the metastatic phenotype, produce and respond to autocrine motility factors from their conditioned media but respond to a lesser extent to platelet-derived growth factor (PDGF). On the other hand, the nontransformed cells do not produce such autocrine factors but respond much more strongly to PDGF than to the transformed cell autocrine factors. There appears therefore to be a change in chemotactic responsiveness that accompanies the transformation of the cell. This alteration may be an important characteristic of the metastatic process.

Significance to Biomedical Research and the Program of the Institute:

A primary feature of tumor cell metastasis is the ability of the cells to locate at target sites distant from the original tumor mass. This requires that these cells, if they spread via the vascular route, e.g., penetrate the endothelium of the vascular system, enter the blood flow, and again penetrate the endothelium from the luminal side to migrate to another target site. At least two processes are involved in traversing basement membrane of the endothelium --

digestion of membrane components and chemotactic migration of the cells. Little is known about either the chemoattractants or the biochemical events that contribute to tumor cell motility. If unique attractants are identified that stimulate the migration of these cells from either the extravascular or luminal sides of the vasculature, it well may be possible to design antagonists that would inhibit migration or develop antibodies that form complexes with the attractant with a similar result. Our results with the conditioned medium attractant may provide a basis to pursue this strategy. Similarly, if the molecular events within the cell are clarified, it may be possible to interfere with some of the post-receptor processes in the motile response.

Proposed Course of Research:

The conditioned media material will be further characterized. If we obtain a homogeneous protein, we will analyze it for its component amino acids and other residues, if any, such as carbohydrates and covalently modified amino acids. We would then attempt sequence determination of the protein and selective enzymatic cleavage to identify the active site. We would then want to determine whether the material is related to known oncogene products. In this connection, we plan to examine the possible temperature-dependent control of the production of a motile phenotype in 3T3 cells injected with a temperature sensitive tumor virus. We would be looking for the regulation of both production of and responsiveness to a chemotactic factor as a function of temperature during cell culture. The pursuit of such studies would contribute to our primary goal of determining whether a correlation exists between transformation and motility characteristics of cells.

Publications:

Terranova, V.P., diFlorio, R., Hujanen, E., Siegal, G., Vasanthakumar, G., Thorgeirsson, U.P., and Schiffmann, E.: Laminin stimulates migration and attachment in rabbit neutrophils. J. Clin. Invest. (in press)

Schiffmann, E., Mandler, R., Katz, D., Murano, G., Liotta, L., Gordon, R., and Chiang, P.K.: Chemotaxis in tumor cells. In Symposium on Metastasis, March 1985, organized by K. Lapis, L. Liotta, and A. Rabson, NCI, NIH, Bethesda, MD. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00892-02 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular biology of the metastatic phenotype

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M.E. Sobel	Senior Investigator	LP, NCI
OTHER:	P.S. Steeg	Guest Resercher	LP, NCI
	L. Kopper	Guest Researcher	LP, NCI
	A.P. Claysmith	Biologist	LP, NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The aim is to investigate the molecular biology of tumor metastasis and invasion. We are using a variety of techniques to identify specific genetic elements whose expression is altered in metastatic cells. Pulse-labeling studies of paired non-metastatic metastatic cells revealed differences in the synthesis of specific proteins. RNA from cultured cell lines and tissues with varying metastatic potential have been analyzed by cell-free translation in a rabbit reticulocyte lysate and by hybridization analysis. In vitro translation studies indicated that the levels of several specific mRNAs are either markedly increased or decreased in metastatic murine melanoma cells. A cDNA library of the murine melanoma cells was constructed. Approximately 40,000 clones of the recombinant DNA library were screened to isolate specific genes involved in the etiology and maintenance of the neoplastic state. To date, we have isolated several molecular clones which code for mRNAs which are expressed to a differential degree in metastatic versus nonmetastatic lines. In addition, levels of mRNA for the major excreted protein (MEP) of transformed murine cells are increased in the nonmetastatic cells. In contrast, levels of mRNA for type IV collagen are increased in the metastatic cells.

GPO 914-918

Project Description

Objectives:

The objective of this project is to determine those genetic elements whose expression distinguishes neoplastic cell behavior from the phenotype of benign tumor cells and normal tissue. Our immediate goals are to:

1. Develop a library bank of DNAs and RNAs from paired cell lines with varying tumorigenic and metastatic potential.

2. Determine the biosynthetic profile of various cell lines and identify specific phenotypic markers of the metastatic cells; i.e. identify proteins whose synthesis is either increased or decreased in metastatic vs. benign vs. normal cells, with particular emphasis on gene products which are uniquely expressed by one cell type.

3. Isolate and characterize recombinant cDNA clones of genes differentially expressed in metastatic cells, with particular attention to connective tissue and basement membrane genes which may be involved in maintaining the metastatic phenotype or may be essential for expression of metastatic behavior.

Methods Employed:

The K1735 mouse melanoma cell line series was previously developed and has been extended by members of the TIM section in collaboration with others. Cell lines studied include benign cells which are tumorigenic but not metastatic in nude mice (clone 16, clone 19) and sister and daughter cell lines with varying metastatic potential (clones M2, M4, TK, EVE, LIV, ADR).

Short time pulse studies of cells grown in vitro were performed to assess unique or differentially synthesized proteins present in metastatic vs. benign vs. normal cells. Total cellular RNA was extracted from cells, translated in vitro in a rabbit reticulocyte lysate, and cell-free translation products were analyzed after polyacrylamide gel electrophoresis and fluorography. Northern hybridization analysis was performed when DNA probes for specific genes were available.

A cDNA library was constructed using RNA template from the metastatic cell line EVE. The cDNAs were inserted into the Pst I site of plasmid pBR322 by GC tailing. Replica filters of the 40,000 cDNA clones were prepared in triplicate. Each set of filters was hybridized to three different P-32 labeled RNA probes representing benign clone 16, benign clone 19, or metastatic cell line EVE. Colonies which hybridized differentially to the benign vs. metastatic RNA were selected for further analysis. The cDNA insert from each selected colony was purified, radioactively labeled, and used as a probe in Northern blots against RNA from each of the K1735 cell lines.

Major Findings:

A library bank of RNAs from cells with varying tumorigenic and metastatic potential has been expanded. It currently consists of multiple preparations from 13 different murine and 20 different human cell lines or tissues.

The biosynthetic profile of these cell lines was determined by pulse-labeling studies of cultured cells and by in vitro translation of RNA. Specific differences in the synthesis of proteins with molecular weights of approximately 17,000, 19,000, 20,000, 40,000, 50,000, 63,000 and 105,000 daltons were detected in the murine series. Their identify is not yet known. These techniques are only sensitive enough to detect differences in cases in which synthesis of a particular peptide represents more than 0.1% of the cellular proteins. It is therefore assumed that other differences exist. Further analysis awaits the development of differential hybridization probes, Northern hybridization analysis, and the use of antibodies directed against specific proteins known to be expressed in metastatic cells.

Northern hybridization analysis of differentially expressed mRNAs in the murine K1735 cell line series was conducted using previously identified cDNA probes. The levels of mRNA for MEP were decreased in the more metastatic cells. MEP is the major excreted protein of transformed mouse cells. It is a mannose 6-phosphate containing glycoprotein with cathepsin B-like properties. Although present in nontransformed cells, its synthesis and secretion by chemically or virally transformed cell lines is enhanced. In contrast, the levels of mRNA for type IV collagen were increased in the more metastatic cells. Type IV collagen is a major component of the basement membrane and is not known to be made in large amounts by melanoma cells.

Of the 40,000 clones in the EVE cDNA library, 23 were selected for further analysis after preliminary screening with RNA from benign vs. metastatic cells. Northern hybridization analysis revealed that 3 clones recognized differentially expressed mRNAs. cDNA clone pNM20 recognizes a large mRNA which is present in greater amount in benign cell lines 16 and 19 than in metastatic cell lines M2, M4, TK, EVE. cDNA clone pNM23 recognizes two mRNAs (4600 bases and 1900 bases) which are also increased in benign cells. The hybridization pattern of cDNA clone pMAP21 is particularly interesting in that it recognizes several differently sized mRNAs even under highly stringent hybridization conditions. In both benign and metastatic cells, pMAP21 recognizes a major mRNA species of approximately 2200 bases. In the benign cells, it also hybridizes to a major mRNA which is approximately 5000 bases long. Hybridization to this size mRNA in metastatic cells is markedly reduced. In contrast, the metastatic cells contain small amounts of three mRNA species (9500, 7100 and 3500 bases long) which are nearly undetectable in the benign cells. It is not yet clear if these species represent unique gene transcripts or are differently spliced mRNAs of the same gene. The cDNA clones are currently being further characterized by restriction endonuclease mapping and DNA sequence analysis.

Significance to Biomedical Research and the Program of the Institute:

The results of our first experiments to detect differences in the biosynthetic profile of metastatic cells indicate that our electic approach to studying the genotype and phenotype of neoplastic cells will be highly successful. The detection of specific proteins and mRNAs whose synthesis is increased or decreased in metastatic cells compared to their more benign (but genetically related) counterparts gives us the capability of developing specific probes to identify those elements which are abnormally present in, or absent from, malignant cells. As our cDNA cloning experiments continue, we expect to ob-

tain sufficient quantities of nucleic acids uniquely expressed in metastatic cells to analyze their sequence and determine controlling elements in the metastatic process. Once identified, it is hoped that these genes and regulatory elements can be manipulated so their expression can be controlled in a more normal manner. Such studies should be instrumental in understanding the biology of cancer cells and may lead to diagnostic and therapeutic advances in malignant disease.

Proposed Course of Research:

Increased emphasis is being placed on isolating more cDNA clones which represent genes differentially expressed by metastatic cells. To this end, a differential cDNA probe is being developed by Rot curve analysis to minimize sequences common to both benign and metastatic cells. These cDNA clones will then be used to probe genomic DNA libraries with the eventual goal of isolating intact genes. Such genes will be introduced into benign cells and the behavior of the transfected cells will be studied to determine if the metastatic phenotype can be genetically transferred.

The potential biological significance of the increased levels of type IV collagen mRNA and the decreased levels of MEP mRNA in the metastatic melanoma cells will be explored. The ability of the cells to actually produce MEP and type IV collagen is currently being ascertained, and the effect of exogenously added protein on cell behavior will be studied. The molecular mechanisms for alterations in mRNA levels will also be explored.

As exemplified by the MEP and type IV collagen examples, we have found it worthwhile to test DNA probes as they become available for their ability to distinguish differentially expressed mRNAs in benign vs. metastatic cells. Future plans include studying oncogene expression as well as expression of plasminogen activator.

Publications:

Doherty, P.J., Hua, L., Liau, G., Gal, S., Graham, D.E., Sobel, M., and Gottesman, M.M.: Malignant transformation and tumor promoter treatment increase levels of a transcript for a secreted glycoprotein. Mol. Cell. Biol. 5: 466-473, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00893-02 LP

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
DNA mediated transfer of metastatic potential

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.J. Muschel	Senior Staff Fellow	LP, NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI
	E.W. Chu	Chief, Cytopathology Section	LP, NCI

COOPERATING UNITS *(if any)*

R. Pozzatti (Postgraduate Fellow) and G. Khoury (Chief), LMV, NCI

LAB/BRANCH
Laboratory of Pathology

SECTION
Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ability of ras^H genes to induce metastatic potential as well as transformation and tumorigenicity was demonstrated in several cell types. NIH-3T3 cells transformed with either the DNA of the Harvey Sarcoma Virus or with the cloned T24 human ras^H oncogene resulted in cells which formed lung metastases. Early passage diploid rat embryo fibroblasts transformed by ras^H (Pozzatti et al) were also metastatic as were early passage Chinese hamster lung fibroblasts transformed by ras^H (Spandidos and Wilkie, Nature (1984) 310: 469). Thus, the ras^H oncogenes can induce metastatic behavior even in diploid fibroblasts.

However, transformation itself or the ability to grow as a tumor was shown to be insufficient to result in metastasis. The normal cellular counterpart of the ras^H oncogene can also transform NIH-3T3 cells if an LTR is placed upstream from the c-ras^H gene to increase the levels of the normal P21 (Chang et al, Nature (1982) 307: 658). The NIH-3T3 cells transformed by this construction, while highly tumorigenic, do not metastasize.

Ras^H did not induce metastatic potential in all recipient cell lines tested. For example C127 cells, a murine epithelioid line which were transformed by ras^H, did not metastasize although morphologically transformed and highly tumorigenic. The induction of metastatic potential in those cells is being attempted in gene transfer experiments.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

Metastasis is a distinct property from tumorigenicity in that tumorigenic cells do not necessarily metastasize. We have recently developed a series of well-defined cells which should allow us to analyze the additional factors which induce metastatic potential. The introduction of the ras^H oncogene into rat embryo fibroblasts, Chinese hamster lung fibroblasts or NIH-3T3 cells leads to transformation, tumorigenicity and metastasis in nude mice. Thus, a single gene in these cells induces metastatic potential. However, this same gene in C127 cells or rat muscle cells while causing transformation and tumorigenicity does not lead to metastasis. We intend to exploit these observations in the following ways.

1. We have shown in collaboration with Rudy Pozzatti, George Khoury and Elizabeth Chu that the transformed rat embryo fibroblasts are either diploid or may have minor karyotypic changes. Since these changes are simple, it will be possible to karyotype metastases and tumors to evaluate the importance of gross chromosomal alterations in contributing to metastasis.

2. Since there are tumorigenic cells containing ras^H which do not metastasize, while ras^H can directly induce metastasis in other cells, we intend to attempt to induce metastasis in these negative cells by DNA mediated gene transfer from the metastatic cells.

3. We intend to investigate the potential of other oncogenes to induce metastasis.

Methods Employed:

$Ca(PO_4)_2$ mediated transfer of DNA, recombinant DNA methods, including gel electrophoresis, Southern blotting and plasmid manipulations. Immunoprecipitation, lung colonization and tumorigenicity assays in nude mice.

Major Findings:

We have found that the ras^H oncogene (either as the Harvey sarcoma virus or the T24 bladder carcinoma gene) when introduced into NIH-3T3 cells results in tumorigenic cells with metastatic potential. The normal cellular counterpart of the ras^H oncogene can also transform NIH-3T3 cells if an LTR is inserted upstream from the $c\text{-}ras^H$ gene to boost expression of the normal P21 (Chang et al, Nature (1982) 307: 658). When cells transformed by a similar construct were tested, they proved to be tumorigenic but nonmetastatic. This finding confirms in this system that tumorigenicity and metastatic capacity are distinct phenotypic properties.

Transformed rat embryo fibroblast (Pozzatti et al, manuscript in preparation) also result in metastasis as do transformed diploid Chinese hamster lung fibroblasts (Spandidos and Wilkie, Nature, ibid.). Thus, diploid early passage cells when transformed by ras^H can become metastatic. This indicates that the unusual properties of NIH-3T3 cells which have been acquired during

many years in culture are not responsible for the induction of metastatic potential by ras^H.

C127 cells transformed by ras^H are highly tumorigenic but are not metastatic. This is also the case for transformed rat muscle cells (Spandidos and Wilkie, ibid.). On the basis of this finding, we speculate that there may exist two complementation groups of genes involved in the development of metastatic potential, one residing in some oncogenes and the other revealed by the difference in the response of the C127 and the NIH-3T3 cells to transformation by v-ras^H.

Significance to Biomedical Research and the Program of the Institute:

This system will allow us to explore the genetic basis of metastasis. Since metastasis is a frequent cause of treatment failure for cancer patients, any understanding of this process which could lead to diagnostic methods to predict metastases or lead to new treatments to prevent metastasis could be of great importance.

Proposed Course of Research:

1. To develop methods to reliably induce metastatic potential in the C127 cells transformed by ras^H including gene transfer, mutagenesis and use of tumor promoters.

2. To determine if other oncogenes will induce metastatic potential.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08266-05 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure and function of basement membrane molecules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C.N. Rao	Visiting Associate	LP, NCI
	U. Wewer	Visiting Fellow	LP, NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI
	I.M.K. Margulies	Biologist	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The nature and assembly of basement membrane constituents namely IV collagen, laminin and heparan sulphate proteoglycan were studied using a variety of in vitro binding assays. These basement membrane macromolecules were isolated from the EHS tumor grown in C57 black mice. Protease-derived fragments of laminin and IV collagen were characterized by rotary shadowing electron microscopy. The domains required for binding of laminin and IV collagen were identified. Laminin is a cross-shaped molecule with three equal short arms and one long arm. The cell binding region of laminin was also identified and found to reside at the intersection of the three short arms. The carbohydrate composition of laminin was obtained and the distribution of sugars on the long and short arms of laminin molecule was studied. Type IV collagen is a rope-like structure (360 nm) with a globular domain at the carboxyterminal end and a disulphide-rich amino terminal end. A major binding site for laminin is identified at about 100 nm away from the globular end of type IV collagen. A complete model has been developed for the orientation of the cell surface laminin receptor, laminin itself, and type IV collagen in the basement membrane.

Project Description

Objectives:

1. To prepare purified protease-derived fragments of basement membrane components. 2. To use these fragments to study the structure and function of basement membrane molecules. 3. To raise polyclonal and monoclonal antibodies to different domains of laminin molecule. 4. To quantitate laminin and fragments of laminin in normal and cancer tissues. 5. To isolate the smallest possible fragment of laminin that has a receptor binding domain.

Methods Employed:

1. Characterization of laminin

Laminin was purified from 0.5 M NaCl extracts of mouse EHS tumor by DEAE cellulose and stored frozen in phosphate-buffered saline, 4.0 M NaCl precipitation.

Protease-derived fragments of laminin were purified and examined by electron microscopy. The alpha fragment generated by digestion with α-thrombin lacks the long arm but retains the three short arms with globular end regions. The C_1 and P_1 fragments generated by chymotrypsin and pepsin, respectively, lack both the long arm and the globular end regions of the short arms. The C_1 fragment has "T" structure with arm lengths of 32 nm. The P_1 fragment is similar in appearance but has shorter arm lengths (26 nm). ^{14}C-IV collagen purified from EHS tissue was digested with pepsin (1:10 w/w) at 4°C for 16 hr. Pepsin destroys the 'NC1' domain of IV collagen leaving a 250 nm major triple helix and the '7S' domain intact.

2. Iodination of laminin and laminin fragments

Laminin and the three fragments were iodinated by the lacto-peroxidase method. The reagents and the unreacted iodine were removed from the iodinated proteins by molecular sieve chromatography.

3. Binding assays

A. To analyze the carbohydrate composition and distribution on laminin - Nitrocellulose filters (13 mm dia. 8 micron pore size Millipore SCWP type) were saturated with 20 µl of the lectin in phosphate-buffered saline (+ calcium + magnesium) pH 7.4 (PBS). Following lectin binding to the nitrocellulose, the filters were immersed in a blocking solution of 3% bovine serum albumin (BSA/PBS: 2 x 90 min) and rinsed with PBS (2 x 30 min). The blocked filters were saturated with ^{125}I-labeled whole laminin or the laminin fragments and incubation was conducted in a humidified chamber at 25°C for 30 min. The filters were washed with PBS (2 x 45 min) and placed in a Packard Gamma Counter to measure the bound radioactivity. The radioactivity bound to NC filters blocked with 3% BSA alone was subtracted as background (8-15%) to give the final value for bound radioactivity. Carbohydrate specificity of the lectin-laminin binding was studied by two means: In the first method, the laminin-lectin binding reaction was conducted in the presence of excess haptenic sugars and in the second

method, the haptenic sugars were added at the end of the binding reaction and the radioactivity was eluted from the filters over 18 hr at 25°C.

B. To study the binding of laminin and IV collagen - ^{14}C-pro IV collagen was soaked overnight in laminin sepharose and the unbound IV collagen was thoroughly washed. The bound IV collagen was successively eluted with 1.0 M NaCl and 6.0 M urea solutions. Pepsin digested IV collagen was also used to quantitate its binding to laminin sepharose column. Iodinated laminin and laminin fragments were employed to bind to IV collagen immobilized on SCWP Millipore filters. Alternatively, labelled IV collagen and its pepsin digest were used to quantitate their binding to laminin and laminin fragments coated onto SCWP Millipore filters.

Major Findings:

1. The nature and location of carbohydrate moieties on the laminin molecule were identified by studying the binding affinity of a series of lectins for purified, protease-derived fragments of laminin. Laminin is a cross-shaped molecule containing 3 short arms (36 nm) and one long arm (76 nm). All arms contain globular end regions by electron microscopy. Purified fragments of laminin were obtained which a) lacked the long arm of the molecule but retained the intact short arms, or b) lacked both the long arm and the globular end regions of the short arms. These two types of fragments differed markedly in lectin binding capacity. Using the known sugar specificities of the lectins and hapten sugar competition for lectin binding to laminin fragments, we conclude the following:

a) α-D-galactosyl end groups are markedly enriched in the globular end regions of the short arms compared to the rod-shaped portions of the molecule.

b) α-D-mannopyranosyl residues are present on both the globular end regions and the rod-shaped portions of the molecule.

c) Exposed N-actely-D-galactosaminyl end groups are absent or present in low amounts on laminin.

d) (NANA)-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→2)-D-Man-terminated oligosaccharide units are enriched on the rod-shaped regions of the short arms compared to the globular end regions.

2. Two monoclonal antibodies to laminin (LAM-I and LAM-II) were used to localize the protease resistant T shaped C_1 or P_1 fragment of laminin. Both LAM-I and LAM-II react with the 200 KD subunit and C_1 fragment suggesting that C_1 fragment originates from the 200 KD subunit. LAM-I but not LAM-II reacts with P_1 fragment suggesting that an epitope for LAM-I lies on the rod-shaped domain of the three short arms while an epitope for LAM-II is present on the globular domains of the short arms. Under mild reducing conditions, about 20% of the 200 KD subunit from laminin and 200 KD subunit retained in α-thrombin digests were identical and different from that of the 400 KD subunit. These data suggest that α-thrombin digestion of laminin can be used to isolate the 200 KD subunit in native form and that the protease resistant T-shaped

intersection of the laminin short arms is embodies in the 200 KD subunit.

3. IV collagen binds laminin with a high affinity and 6.0 M urea was needed to elute the IV collagen bound to laminin. Binding assays demonstrated that the end globular domains on laminin short arms binds the pepsin sensitive carboxy terminal 'NCI' domain of IV collagen. The long arm of the laminin molecule binds the heparan sulphate proteoglycan.

Significance to Biomedical Research and the Program of the Institute:

Invasive neoplasia is accompanied by profound changes in the structure and amount of basement membranes. Study of the structure of basement membrane components and their interaction with cells may lead to therapeutic approaches which involve modification of tumor cell interaction with basement membranes.

Proposed Course of Research:

1) To chemically characterize the cell binding domains of laminin; 2) to characterize the binding domains on the NC1 domain type IV collagen molecule; 3) to study the assembly of basement membranes in vitro; and 4) to visualize (by EM) the binding of laminin, type IV collagen and proteoglycan together.

Publications:

Barksy, S.H., Rao, C.N., Williams, J.E., and Liotta, L.A.: Laminin molecular domains which alter metastasis in a murine model. J. Clin. Invest. 74: 843-848, 1984.

Roberts, D.D., Rao, C.N., Magnani, J.L., Spitalnik, S.L., Liotta, L.A., and Ginsburg, V.: Laminin binds specifically to sulfated glycolipids. Proc. Natl. Acad. Sci. USA 82: 1306-1310, 1985.

Rao, C.N., Margulies, I.M.K., and Liotta, L.A.: Binding domain for laminin on type IV collagen. Biochem. Biophys. Res. Commun. 128: 45-52, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09127-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Purification and biochemical control of type IV collagenase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Turpeenniemi-Hujanen	Visiting Fellow	LP, NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI
	U.P. Thorgeirsson	Visiting Scientist	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Type IV collagenase is an important basement membrane degrading metalloproteinase discovered by our laboratory. This enzyme is produced by malignant cells to facilitate their traversation through blood vessel walls as well as during transition from in situ to invasive carcinoma. Human type IV collagenase was isolated from culture supernatants of A2058 metastatic melanoma cells after four steps of purification. The collagenase has a molecular weight of 70 KDa and produced a typical cleavage pattern of type IV collagen by SDS gel electrophoresis. A second polypeptide was eluted off the collagen IV affinity column which has a molecular weight of 100 KDa. This new collagen IV binding protein has no collagenolytic activity and does not possess any characteristics of known matrix proteins.

The control mechanism for type IV collagenase was studied in vitro in relation to attachment proteins such as laminin and fibronectin, as well as growth factors. The presence of laminin in the culture medium stimulated type IV collagenolytic activity of A2058 melanoma cells 200%, but fibronectin and BSA had no effect. The collagenase stimulating effect was mediated through the cell binding part of laminin, as demonstrated by the P-1 fragment of laminin which also was stimulatory (600%) when added to the culture supernatants. The stimulating effect was abolished by monoclonal antibodies to the laminin receptor, that specifically inhibit the cell binding site. In contrast to the laminin effect, three growth factors added to the A2058 culture supernatants inhibited the collagenase IV activity. The maximum inhibitory effect measured with EGF was 50%, FGF 43% and transferrin 30%. These in vitro studies show that production and/or secretion of type IV collagenase can be modulated by the cell binding fragment of the laminin molecule as well as by different growth factors.

Project Description

Objectives:

1. To purify type IV collagenase from tumor cell supernatants and raise monoclonal and polyclonal antibodies to the collagenase.

2. To study the control mechanism of type IV collagenase in relation to a) tumor cell attachment and b) growth factors.

Methods Employed:

Type IV collagenase was purified from culture supernatants of a human metastatic melanoma cell line, A2058. Serum-free supernatants from 20 subconfluent roller flasks were collected for 4 days. The cells were fed with serum-containing medium for two days in between, i.e. after the first two days of collection. The 6 liters of collected supernatants were first concentrated 40-fold with Amicon ultrafiltration, then further concentrated 10-fold by ammonium sulfate precipitation (0-60%), followed by overnight dialysis (0.2 M NaCl, 0.05 M Tris-HCl, pH 7.4) at 4°C. The debris was removed by centrifugation and the concentrated supernatant stored at -70°C until the second step of purification was started, i.e. molecular sieve chromatography (Bio-Gel A - 1.5 M). The supernatant was passed through the Bio-Gel and fractions collected, using the same Tris buffer as used for the dialysis. The fractions showing the highest collagenase IV activity were pooled and applied to a collagen IV Sepharose 4B affinity column. The type IV collagen used for the affinity chromatography was purified from an EHS tumor as described by Orkin et al. (J. Exp. Med. 145: 204, 1977). The purity of the type IV collagen was verified by SDS-PAGE. The affinity column was eluted with 50% ethylene glycol in the regular Tris-buffer. Protein concentration was measured in the eluted fractions, and the fractions containing the maximum amounts of protein were pooled. The affinity purified material contained two polypeptides that were separated by SDS-PAGE, cut out and then eluted from the gel.

Assay of type IV collagen degrading metalloprotease - The secreted type IV collagen-degrading metalloprotease activity was measured in serum-free cell culture medium, which was concentrated a hundred fold with ammonium sulfate precipitation (0-60%) followed by dialysis against 0.2 M NaCl and 0.05 M Tris-HCl (pH 7.4 at 4°C). The type IV collagen degradation was assayed as described previously with the use of soluble [^{14}C] proline-labeled type IV collagen as a substrate. The enzyme of each sample was activated by the addition of 10 µg trypsin/ml for 10 minutes at 37°C and then assayed in the presence of 50 µg soybean trypsin inhibitor/ml, 3.8 mM N-ethylmaleimide, and 1,000 kallikrein inhibiting units of aprotinin/ml for 6 hours at 37°C. Parallel samples were assayed in the presence of 10 mM EDTA to ensure that only the metalloprotease activity was tested. Incubations were also done with and without trypsin in the absence of the enzyme. The reaction was terminated by the incubation of the sample at 0-4°C for 30 minutes in the presence of EDTA (10 mM), trichloroacetic acid (0.6%), and tannic acid (0.03%). The undigested substrate was removed by centrifugation, and the radioactivity of the supernatants was measured in a β-scintillation counter. The amount of degraded type IV collagen was then calculated from the specific activity of the radiolabeled substrate. The

cells were counted after 4 days' collection of culture supernatants, and the DNA was quantitated as described previously. The enzyme activity was expressed as the amount of type IV collagen degradation per 10^6 cells and per total cellular DNA. All the assays were performed with an amount of substrate that was within the linear range of the assay by use of a series of cell numbers between 0.5×10^6 and 5×10^6 cells for each sample.

Binding affinity between the 100 KD polypeptide and various other proteins was performed on nitrocellulose. The nitrocellulose filters were impregnated with BSA, collagen types I, II and III, laminin, fibronectin, heparan sulfate and chondroitin sulfate proteoglycan. The nonspecific binding sites were blocked with 1% gelatin buffer, the filters were then washed in PBS and incubated with the iodinated 100 K protein for 16 hr. The filters were finally washed extensively in PBS and the radioactivity measured in a gamma counter.

Major Findings:

Purification of tumor cell type IV collagenase was achieved in four steps, i.e. ammonium sulfate precipitation, molecular sieve chromatography, type IV collagen affinity chromatography, and gel electrophoresis. Type IV collagenase activity was present, although decreased after the three first steps of purification. The peak enzyme activity in the molecular sieve chromatography was corresponding to the molecular weight of about 70.000. The material eluted from type IV collagen affinity column was shown by SDS-PAGE to contain two polypeptides with molecular weights of 70.000 and 100.000. The two polypeptides were cut out and eluted electrophoretically from the SDS gel. Polyclonal antibodies were raised in rabbits against each polypeptide. The 70 KD antibody inhibited type IV collagenase activity of the crude A2058 enzyme preparation up to 30%, while the 100 KD antibody was not inhibitory in the same assay. The second piece of evidence that the 70 KD was the type IV collagenase came from digestion experiment with trypsin, which degraded 100 KD protein to small polypeptides but caused only a slight change in molecular weight of the 70 KD protein. Trypsin treatment is required of type IV collagenase activation and has been shown to cause only a small conversion of the molecular weight of this enzyme (Sato, T. et al., J. Biol. Chem. 258: 3058, 1983). Type IV collagen was typically cleaved into 1/4 and 3/4 fragments by the crude A2058 enzyme as demonstrated by SDS PAGE. Reduction of the 70 KD polypeptide did not change its mobility in SDS-PAGE, which indicates that the enzyme does not contain inter- or intramolecular disulfide bonds. The 100 KD protein was tested for binding affinity to various matrix proteins as well as for protease susceptibility. As expected, the 100 KD protein bound effectively to type IV collagen, reaching saturation at 8 µg/ml for 5 µg of type IV collagen. Comparatively, affinity for BSA and type I collagen was 20%, and fibronectin and laminin 30% of the binding to type IV collagen. Background readings were found with collagen types II and III, heparan sulfate proteoglycan and chondroitin sulfate proteoglycan. The findings on the protease susceptibility excluded the possibility that the 100 KD protein is a fragment of type IV collagen, i.e. it was degraded by trypsin, chymotrypsin, pepsin and V-8 protease but not by bacterial collagenase. Furthermore, the 100 KD protein did not cross-react with monoclonal antibodies against laminin when tested in a peroxidase-linked biotin avidin system. Like the 75 KD protein, the 100 KD protein did not change the mobility in SDS-PAGE under reduced conditions.

The effect of laminin and growth factors was examined on the expression of type IV collagenase by three tumor cell lines in culture. Laminin, fibronectin and BSA (4 µg/ml) were added in a serum-free medium to subconfluent cultures of the tumor cell lines and incubated for 24 hr. Laminin stimulated type IV collagenase activity 200% but fibronectin and BSA had no effect. Experiments with different concentrations of laminin and BSA showed that the increase in collagenase caused by laminin was dose dependent. BSA served as a negative control. Incubation with an inhibitor of protein synthesis (cycloheximide) showed that the type IV collagenase activity was dependent on protein synthesis. In the absence of laminin, the enzyme activity was reduced 95% by cycloheximide, and in the presence of laminin the enzyme activity was reduced 70%. These findings suggest that the laminin effect on type IV collagenase is partially due to increased secretion of stored enzyme sources. The stimulating effect of laminin on the collagenolytic activity was also demonstrated on two other tumor cell lines, i.e. HT-1080 fibrosarcoma (170%) and B16-F10 melanoma (270%). To further investigate whether the cell binding part of laminin is responsible for the collagenolytic stimulation, we tested the P1 fragment of laminin that binds to the laminin receptor on the tumor cell surface. The P1 fragment of laminin added to the culture medium in equivalent molarity to laminin stimulated (580%) the collagenase. This stimulatory effect of laminin molecule was abolished by monoclonal antibodies to laminin that specifically inhibit the cellular binding sites. The effect of growth factors on type IV collagenolytic activity of culture supernatants of A2058 cells was studied in a similar manner to the laminin. All three growth factors studied had mild inhibitory effect on the secreted type IV collagenase activity. The maximum inhibitory effect with EGF at a concentration of 20 ng/ml was 50%, for FGF (34 ng/ml) 43%, and transferrin (10 µg/ml) 20%.

Significance to Biomedical Research and the Program of the Institute:

Reports from different research groups have demonstrated strong correlation between type IV collagenolytic activity of tumor cells and their metastatic behavior. The metastatic process is highly complex and for successful completion of this process, a coordination of many cellular and host factors is required. A number of proteolytic enzymes have been associated with tumor invasion. We believe that type IV collagenase that specifically breaks down basement membrane collagen is an important enzyme that facilitates tumor cell invasion through vascular walls. Continued purification of tumor cell collagenase IV and development of monoclonal and polyclonal antibodies will open the door to numerous genetic and biochemical studies. In comparing the normal and tumor cell collagenase IV, detection of some antigenic differences could lead to development of specific monoclonal antibodies that would only react with the tumor cell collagenase IV but not with inflammatory cell collagenase. The use of monoclonal antibodies and specific protease inhibitors against type IV collagenase in cancer treatment may be potentially important for intervention of the metastatic process.

Proposed Course of Research:

1. Continued purification of type IV collagenase from the A2058 melanoma culture supernatants. The enzyme will first be used for raising monoclonal antibodies in collaboration with Dr. Pat Hand, NCI. Polyclonal antibodies will also be made. Subsequently, a radioimmunoassay will be developed for detection of type IV collagenase in different types of tissues and fluids.

2. In collaboration with Dr. Garbisa, the tumor cell enzyme will be compared with type IV collagenase purified from activated human macrophages in Dr. Garbisa's laboratory.

3. After sufficient amount of the tumor cell collagenase IV has been purified and specific antibodies developed, the gene for type IV collagenase will be cloned. The cloning will be done in collaboration with another research group.

4. Studies on the biochemical control mechanism for type IV collagenase will be continued, especially the effect of cell attachment and laminin binding to the cell surface receptor. Besides in vitro experiments, the effect of laminin and other proteins on tumor secreted collagenase IV will also be studied in vivo. Subcutaneous chambers placed in close contact with the transplanted tumors will collect secreted tumor fluids that subsequently will be withdrawn and analyzed.

5. Further characterization of the 100 KD collagen IV binding protein will be done by Dr. Turpeenniemi-Hujanen after her return to Finland.

6. Sequence the type IV collagen cleavage site.

Publications:

Turpeenniemi-Hujanen, T., Thorgeirsson, U.P., and Liotta, L.A.: Collagenases in cellular extravasation. Ann. Rep. Med. Chem. 19: 231-239, 1984.

Thorgeirsson, U.P., Turpeenniemi-Hujanen, T., and Liotta, L.A.: Cancer cells, components of basement membranes, and proteolytic enzymes. Int. Rev. Exp. Pathol. 27: 203-225, 1985.

Turpeenniemi-Hujanen, T., Thorgeirsson, U.P., Hart, I.R., Grant, S.S., and Liotta, L.A.: Expression of collagenase IV (basement membrane collagenase) activity in murine tumor cell hybrids which differ in metastatic potential. J. Natl. Cancer Inst. vol. 74, 1985 (in press)

Thorgeirsson, U.P., Turpeenniemi-Hujanen, T., and Liotta, L.A.: Mechanisms of tumor invasion and their potential therapeutic modifications. In Mihich, E. (Ed.): Biological Responses in Cancer, Vol. 4 (in press)

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Smith, H.S., Liotta, L.A., Hancock, M.C., Wolman, S.R., and Hackett, A.J.: Invasiveness and ploidy of human mammary carcinomas in short-term culture. Proc. Natl. Acad. Sci. USA 82: 1805-1809, 1985.

Reichert, C.M., Costa, J.C., Barsky, S.H., Claysmith, A.P., Liotta, L.A., Enzinger, F.M., and Triche, T.J.: Pachydermodactyly. Clin. Orthopaed. Rel. Res. 194: 252-257, 1985.

Ognibene, F.P., Steis, R.G., Macher, A.M., Liotta, L.A., Gelmann, E., Pass, H.I., Lane, H.C., Fauci, A.S., Parrillo, J.E., Masur, H., and Shelhamer, J.H.: Kaposi's sarcoma causing pulmonary infiltrates and respiratory failure in the acquired immunodeficiency syndrome. Ann. Intern. Med. 102: 471-475, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09130-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Laminin receptor in breast tissue, benign and malignant tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G.J. Bryant	Expert	LP, NCI
OTHER:	C.N. Rao	Visiting Associate	LP, NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI

COOPERATING UNITS *(if any)*

R.R. Brentani, Ludwig Institute for Cancer Research, Sao Paulo, Brazil

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Laminin, a major glycoprotein of basement membranes, exhibits saturable binding to the surface of certain neoplastic and normal cells. Examination of various cell types (i.e. human pancreatic carcinoma, melanoma and bladder carcinoma) via live cell binding techniques indicate the number of receptors to be 50-110,000 per cell depending on cell type. In our laboratory, the laminin receptor has been isolated from human breast carcinoma cells and tissue and mouse melanoma cells. The receptor and subsequent ligand binding have been implicated to play an important role in tumor cell attachment, one of the steps in tumor invasion and metastasis. The proposed study is designed to measure and correlate laminin receptor binding capacity of breast tissues with clinical information. Laminin receptor binding capacity markedly differs between benign, malignant and normal human breast tissue. Preliminary studies indicate a 50-fold increase in specific laminin binding activity in malignant versus benign breast tissues. These findings suggest a site for possible intervention in the sequence of tumor invasion.

GPO 914-816

Project Description

Objectives:

1. Establish a standard protocol for collection of both human breast tissue and pertinent clinical data.

2. Determine specific laminin binding capacity of normal, benign and malignant human breast tissues.

3. Correlate the above results with clinical data.

Methods Employed:

Specific laminin binding capacity of normal, benign and malignant human breast tissue will be determined via assay of ^{125}I-laminin binding to plasma membranes of breast tissues in the presence and absence of excess unlabeled laminin.

Major Findings:

High affinity laminin binding has been demonstrated in both human breast carcinoma cells and the plasma membrane of human breast tissues. The laminin receptor isolated from both breast tissue and cells has been demonstrated as a single band via gel electrophoresis with a molecular weight of approximately 67,000. Preliminary studies indicate a significant difference in specific laminin binding capacity of human invasive breast carcinoma plasma membranes compared to normal or benign breast tissues.

Significance to Biomedical Research and the Program of the Institute:

Laminin receptor binding may facilitate metastasis of tumor cells. The preliminary findings are supportive of the hypothesis that there are a large number of available receptors (i.e. without ligand) on invading tumor cells which may facilitate interaction of these cells with host membrane(s) during metastasis. If, in fact, this is the case, then one could envision an area of possible therapeutic intervention which would attempt to manipulate the availability of the receptors.

Proposed Course of Research:

Establish specific laminin binding assay of breast carcinoma plasma membranes as a technique and correlate the results with clinical information.

Publications:

Liotta, L.A., Rao, C.N., and Barsky, S.H.: Tumor invasion and the extracellular matrix. Lab. Invest. 49: 636-649, 1983.

Barsky, S.H., Rao, C.N., Hyams, and Liotta, L.A.: Characterization of a laminin receptor from human breast carcinoma tissue. Breast Cancer Res. Treat. 4: 181-188, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09131-01 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*

Molecular cloning of connective tissue matrix molecules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M.E. Sobel	Senior Investigator	LP, NCI
OTHER:	U. Wewer	Visiting Fellow	LP, NCI
	A.P. Claysmith	Biologist	LP, NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI

COOPERATING UNITS *(if any)*

Dr. M. Jaye, Meloy Lab., Springfield, VA; Dr. F. Ramirez, Univ. of Medicine and Dentistry of N.J.-Rutgers Med. School, Piscataway, NJ; Dr. G. Vogeli, LMDBI, NEI

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The interaction of the tumor cell with its extracellular matrix may play an important role in determining its metastatic and invasive properties. To better understand the protein components that make up the extracellular matrix and how they are regulated, we have undertaken to construct, isolate, and characterize molecular clones of laminin receptor and of several different collagens. Laminin receptor is a cell surface protein to which laminin (a major component of basement membrane) specifically binds. Using a monoclonal antibody directed against the laminin-binding domain of laminin receptor, we screened a human endothelial cell cDNA λgt11 library. Six plaques showed an intense reaction with the anti-laminin receptor monoclonal but showed no reactivity toward a variety of control antibodies. The sizes of the cDNA inserts of the six clones ranged from 450 to 950 base pairs. Restriction enzyme mapping and Southern hybridization identified a 400 base pair fragment which was identical in each cDNA clone, suggesting that this fragment may represent the laminin-binding domain of laminin receptor. The putative laminin receptor cDNA clone recognizes a 1700 base mRNA, which would be sufficient in length to code for a protein with the expected size of laminin receptor. In addition, cDNA clones coding for mouse type IV collagen and genomic clones coding for human type II collagen have been characterized.

GPO 914-918

Project Description

Objectives:

The objective of this project is to molecularly clone specific components of the extracellular matrix with which cells interact. Particular emphasis is placed on proteins that comprise or interact with the basement membrane. The integrity or lack thereof of the basement membrane surrounding tumors may be an important indicator of its essential invasiveness. Our immediate goals are to:

1. Isolate and identify a cDNA clone encoding laminin receptor, a protein on the cell surface to which laminin specifically binds.

2. Determine the protein sequences of laminin receptor by sequencing the cDNA clone. This will increase the understanding of the structure of the laminin receptor.

3. Study the regulation of laminin receptor synthesis and expression.

4. Isolate genomic clones and the promoter of the laminin receptor gene. Transfect the entire gene into cells to determine the effect of laminin receptor on cell behavior, specifically the ability of a cell to attach to basement membrane or to modulate its invasive and metastatic properties.

5. Complete characterization of cDNA clones encoding type IV collagen, the major component of basement membrane.

Methods Employed:

Laminin receptor was previously purified from the plasma membranes of metastatic breast carcinoma tissue and monoclonal antibodies were obtained and characterized by members of the Tumor Invasion and Metastases Section in collaboration with others. One such antibody, 2H5, specifically recognized laminin receptor on Western blots and could block binding of laminin to either plasma membranes or cells, or could block attachment of cells to amnion basement membrane. It presumably recognizes the laminin-binding domain of laminin receptor.

A cDNA library was constructed at Meloy Laboratories using RNA template from human endothelial cells. The cDNAs were inserted into the EcoR1 site of lambda phage gt11 in such a way that the cDNA was fused to the end of the β-galactosidase gene. In this expression library, depending on orientation and reading frame, one in six phages could synthesize a fusion protein consisting of β-galactosidase and the protein fragment encoded by the cDNA. 1.5 million plaques of the recombinant λgt11 library were screened using the monoclonal antibody 2H5. Positive plaques were purified and tested for nonreactivity toward control antibodies. They were analyzed by restriction endonuclease mapping and Southern hybridization.

The level of mRNA recognized by the cDNA insert was determined in a variety of tissues or cells by Northern hybridization.

Major Findings:

Preliminary characterization of a laminin receptor cDNA clone. Six plaques were selected after screening of the human endothelial cell cDNA λ-gt11 library with the monoclonal antibody directed against the laminin binding domain of the laminin receptor. After plaque purification, all six clones showed an intense reaction with the anti-laminin receptor monoclonal antibody. The clones showed no reactivity toward a class-matched monoclonal antibody directed against human α-amylase, nor toward antibodies directed against proteins similar in size to laminin receptor, e.g. albumin. The sizes of the cDNA inserts of the six clones ranged from 450 to 950 base pairs. Restriction enzyme mapping and Southern hybridization identified a 400 base pair fragment which was identical in each cDNA clone. The coding sequence of this fragment may represent the antigenic domain recognized by the anti-laminin receptor monoclonal antibody. The 400 base pair fragment was nick translated and hybridized to RNA from a variety of human cell lines whose ability to bind to laminin had been previously studied. The cDNA probe recognized a 1700 base mRNA which would be sufficient in length to code for a protein with the expected size of laminin receptor. The level of hybridized RNA from each cell type correlated with the laminin binding assays. Definitive identification of the clones awaits protein sequencing data and will be accomplished by comparison of the cDNA-predicted protein sequence with the protein sequence of the purified laminin receptor.

Characterization of an alpha 1 type IV collagen cDNA clone. DNA sequence data from a previously isolated type IV collagen cDNA clone established that the cDNA encodes 270 amino acids of a helical portion of the alpha 1 (IV) collagen chain. As expected for a type IV (basement membrane) collagen, there are interruptions of the helical gly-X-Y repeat unit in the coding sequence. In addition, two amino acid stretches, one of 10 amino acids and the other of 7 amino acids, are repeated within the sequence.

Characterization of the human type II collagen gene. Using a cDNA probe previously identified as encoding the cartilage-specific type II procollagen, a series of genomic clones were isolated from a human genomic library. The overlapping genomic clones contain 45 kilobases of contiguous human DNA. The actual human type II collagen is 30 kilobases in size. Comparison to other fibrillar collagens demonstrates the marked conservation of gene structure through the evolution of the interstitial collagen gene family. The gene has been localized to chromosome 12.

Significance to Biomedical Research and the Program of the Institute:

The interaction of highly metastatic tumor cells with the basement membrane is fundamentally different from the interaction of benign cells. The identification of a specific cell surface receptor to which laminin binds is a significant breakthrough in understanding of these interactions. Progress has been limited due to the difficulty in isolating sufficient quantities of laminin receptor. The molecular cloning of laminin receptor will provide us with sequencing data that will circumvent this problem. The ability to probe cells for mRNA content and to eventually obtain regulatory elements of the laminin receptor gene will also provide us with tools to understand how the synthesis of laminin receptor is regulated. Similarly, characterization of a cDNA clone

for type IV collagen has enabled us to study the increased synthesis of this basement membrane component in metastatic cells (see project Z01 CB 00892-01 LP), once again highlighting the importance of extracellular matrix in neoplastic disease.

Proposed Course of Research:

Primary emphasis is being placed on definitive identification of the putative laminin receptor cDNA clones. In addition to DNA sequence, attempts are directed toward demonstrating that the clones encode a protein with laminin-binding activity. The synthesis of laminin receptor mRNA will be studied in a variety of cell systems to determine if expression of the receptor can be modulated at a transcriptional level. Genomic DNA libraries will be screened to isolate the laminin receptor gene. This will eventually permit us to transfect the gene into cells which have low levels of expressed laminin receptor to determine the effect of the receptor on cell behavior.

The characterization of the type II and type IV collagen clones is essentially complete. Further work on these will be conducted by our collaborators, enabling us to direct our full resources toward the laminin receptor clones.

Publications:

Young, M.F., Vogeli, G., Nunez, A.M., Fernandez, M.P., Sullivan, M., and Sobel, M.E.: Isolation of cDNA and genomic DNA clones encoding type II collagen. Nucl. Acids Res. 12: 4207-4228, 1984.

Fernandez, M.P., Young, M.F., and Sobel, M.E.: Methylation of type II and type I collagen genes in differentiated and dedifferentiated chondrocytes. J. Biol. Chem. 260: 2374-2378, 1985.

Sangiorgi, F.O., Benson-Chanda, V., deWet, W.J., Sobel, M.E., Tsipouras, P., and Ramirez, F.: Isolation and partial characterization of the entire human proα1(II) collagen gene. Nucl. Acids Res. 13: 2207-2225, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00550-05 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

Immunologic characterization of malignant lymphomas

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E.S. Jaffe	Chief, Hematopathology Section	LP, NCI
OTHER:	J. Cossman	Senior Investigator	LP, NCI
	D.L. Longo	Senior Investigator	MB, NCI
	L.A. Matis	Senior Investigator	MB, NCI
	L.M. Neckers	Expert	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

In order to assess the clinical and pathologic significance of the immunologic characterization of human malignant lymphomas, fresh biopsy tissues are obtained from patients referred to the Clinical Center for treatment. Biopsies are obtained with patient permission prior to therapy and processed in the Hematopathology Section. The neoplastic cells are characterized as to their origin from T cells, B cells, or histiocytes, and in addition can be identified as belonging to specific developmental and functional subpopulations. This data is then correlated with clinical and pathologic data.

This information is utilized to develop improved classifications of disease and to distinguish new clinicopathologic entities. It also will be used as a basis for potential immunotherapy or adjunctive immunotherapy in a program of autologous bone marrow transplantation.

Project Description

Objectives:

To determine the clinical importance of immunologic phenotype, and determine if it correlates with clinical presentation, stage, response to therapy or survival. To determine the correlation of immunologic phenotype with conventional morphology. To improve diagnosis and subclassification of human malignant lymphomas.

Methods Employed:

Cells derived from malignant lesions are analyzed with a large battery of monoclonal antibodies directed against lineage-restricted surface and cytoplasmic epitopes, surface receptors and growth factor receptors. Cells are analyzed using a fluorescence activated cell sorter which permits collection of quantitative data as to density of surface epitopes and permits distinction of heterogeneous cell populations in tumor cell suspensions. Cells are also analyzed using immunocytochemical methods in both frozen sections and cytocentrifuge smears. This permits morphologic correlations to be drawn and allows for topographical distribution of cells within tissues.

Cells are also studied for a variety of lysosomal enzymes which are particularly useful in identifying cells of mononuclear phagocytic origin.

Patients studied are clinically and pathologically staged and treated in a uniform manner on NCI protocols.

Currently being developed are techniques of in-situ hybridization to study oncogene expression of normal and neoplastic lymphoid cells, to be correlated with phenotypic characteristics.

Major Findings:

Almost all low-grade lymphomas are of B-cell origin and can be related to sequential stages of normal B-cell maturation. Subtypes of low-grade B-cell lymphomas can be distinguished phenotypically and such phenotypes correlate with morphologic subtypes previously described.

Diffuse aggressive non-Hodgkin's lymphomas are phenotypically heterogeneous and surface phenotype in general cannot be accurately predicted by morphologic parameters. Diffuse aggressive lymphomas of both B- and T-cell types are equally responsive to aggressive chemotherapy regimens.

Terminal deoxynucleotidyl transferase (TdT) activity is uniquely associated with lymphoblastic malignancies and is not seen in other forms of non-Hodgkin's lymphoma. Immunocytochemical methods for the detection of TdT are more accurate and specific than biochemical methods.

Lymphoblastic malignancies are derived from early T- and B-lymphocytes. Although lymphoblastic lymphomas (LBL) of precursor B cells are uncommon, this phenotype may be seen in up to 20% of cases. Such cases have differed clinically

from pre-T LBL in that they have not been associated with mediastinal masses and have presented with either lytic bone lesions or generalized lymphadenopathy.

B-cell non-Hodgkin's lymphomas with large numbers of infiltrating normal T cells have been described. Such cases mimic peripheral T-cell lymphomas morphologically and may be confused with peripheral T-cell lymphomas phenotypically if minor subpopulations of monoclonal B cells are overlooked. Clinically, these cases have been associated with an indolent clinical course, although histologically they fall into the diffuse aggressive subtypes.

Hairy cell leukemia is a B-cell neoplasm with a distinctive antigenic phenotype. The cells strongly expresses Tac, the receptor for T-cell growth factor.

A subclassification of mature or post-thymic T-cell malignancies has been proposed which delineates distinctive clinicopathologic entities with differing clinical presentations and prognoses. Phenotypic and functional correlations have been drawn.

The neoplastic cells of Hodgkin's disease stain with Leu M1, which does not stain non-Hodgkin's lymphomas of either B- or T-cell origin. Because the antibody can be applied to routinely processed paraffin-embedded sections, it is particularly useful in differential diagnosis. An exception is the nodular subtype of lymphocyte predominant Hodgkin's disease, which lends support to the concept that this subtype differs pathogenetically from the other subtypes of Hodgkin's disease.

The neoplastic cells of Hodgkin's disease also stain with anti-Tac (receptor for IL-2), OKT9 (transferrin receptors), anti-Leu 10, and anti-HLA-DR, but fail to stain with antibodies specific for T or B lymphocytes.

Both lymphoid and epithelial components of thymomas mirror the phenotype of normal thymus gland. The predominant epithelial cell has neuroendocrine features. The thymocytes, although predominantly cortical in phenotype, do show cortical-medullary differentiation.

Significance to Biomedical Research and the Program of the Institute:

This information will affect future development of clinical protocols, as tumors of differing immunotypes may require different therapies. This has already been shown to be true for lymphoblastic lymphoma. Neoplastic expansions often permit the identification of normal cellular phenotypes not previously recognized, and lead to increased understanding of the immune system.

Proposed Course of Research:

New lymphoma patients accrued to the NCI will be prospectively studied. Patients with recurrent disease will be studied to address questions of constancy of phenotype, clonal evolutions, and multiple neoplastic clones. Techniques for in-situ hybridization are being developed so that phenotypic parameters may be correlated with oncogene expression. Molecular probes will also be used for molecular characterization of immunologic phenotype. Phenotypic information will be utilized in a collaborative program with Dr. R. Steis

and other co-investigators as a basis for adjunctive immunotherapy for autologous bone marrow transplantation.

Publications:

Magrath, I.T., Janus, C., Edwards, B.K., Spiegel, R., Jaffe, E.S., Berard, C.W., Miliauskas, J., Morris, K., and Barnwell, R.: An effective therapy for both undifferentiated (including Burkitt's) lymphomas and lymphoblastic lymphomas in children and young adults. Blood 63: 1102-1111, 1984.

Cossman, J., Jaffe, E.S., and Fisher, R.I.: Immunologic phenotypes of diffuse, aggressive, non-Hodgkin's lymphomas. Correlation with clinical features. Cancer 54: 1310-1317, 1984.

Jaffe, E.S.: Post-thymic lymphoid neoplasia. Chapter 9. In Jaffe, E.S. (Ed.): Surgical Pathology of Lymph Nodes and Related Organs. Series: Major Problems in Pathology. Philadelphia, W.B. Saunders, 1985, pp. 218-248.

Hsu, S.-M. and Jaffe, E.S.: Phenotypic expression of B-lymphocytes. 1. Identification with monoclonal antibodies in normal lymphoid tissues. Am. J. Pathol. 114: 387-395, 1984.

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Jaffe, E.S.: Pathologic and clinical spectrum of post-thymic T-cell malignancies. Cancer Invest. 2: 413-426, 1984.

Martin, S.E., Zhang, H.-Z., Magyarosy, E., Jaffe, E.S., Hsu, S.-M., and Chu, E.W.: Immunological methods in cytology: Definitive diagnosis of non-Hodgkin's lymphomas using immunological markers for T and B cells. Am. J. Clin. Pathol. 82: 666-673, 1984.

Murphy, S. and Jaffe, E.S.: Editorial Retrospective: Terminal transferase activity and lymphoblastic neoplasms. N. Engl. J. Med. 311: 1373-1375, 1984.

Hsu, S.-M., Yang, K., and Jaffe, E.S.: Phenotypic expression of Hodgkin's and Reed-Sternberg cells in Hodgkin's disease. Am. J. Pathol. 118: 209-217, 1985.

Jaffe, E.S.: An overview of the classification of non-Hodgkin's lymph nodes and related organs. Chapter 5. In Jaffe, E.S. (Ed.): Surgical Pathology of Lymph Nodes and Related Organs. Series: Major Problems in Pathology. Philadelphia, W.B. Saunders, 1985, pp. 135-145.

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Braziel, R.M., Hsu, S.-M., and Jaffe, E.S.: Lymph nodes, spleen, and thymus. In Spicer, S.S., Garvin, A.J., and Hennigar, G.R. (Eds.): Application of Histochemistry to Pathologic Diagnosis. New York, Marcel Dekker, Inc. (in press)

Braziel, R.M., Sussman, E., Neckers, L.M., Jaffe, E.S., and Cossman, J.: Induction of immunoglobulin secretion in follicular non-Hodgkin's lymphomas: Role of immunoregulatory T cells. Blood (in press)

Boccia, R.V., Longo, D.L., Lieber, M.L., Jaffe, E.S., and Fisher, R.I.: Multiple recurrences of acute tumor lysis syndrome in an indolent non-Hodgkin's lymphoma. Cancer (in press)

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Jaffe, E.S. and Cossman, J.: Immunodiagnosis of lymphoid and mononuclear phagocytic neoplasms. In 3rd Edition, Manual of Clinical Laboratory Immunology. American Society for Microbiology, Washington, D.C. (in press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00551-05 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Stimulation of phagocytosis by a peripheral T-cell lymphoma-derived lymphokine

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E.S. Jaffe	Chief, Hematopathology Section	LP, NCI
OTHER:	C.R. Simrell	Senior Assistant Surgeon	LP, NCI
	A.S. Fauci	Chief	LIR, NIAID
	J.D. Margolick	Medical Staff Fellow	LIR, NIAID
	E.H. Lipford	Expert	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Certain patients with malignant lymphomas originating from peripheral T cells develop a rapidly fatal syndrome which mimics malignant histiocytosis. It is suspected that the pathogenetic mechanism of this phenomenon may involve a lymphokine produced by the neoplastic T cell which can stimulate the phagocytic cells of the reticuloendothelial system. In order to test this hypothesis, neoplastic cells from fresh biopsies of patients with malignant lymphoma are placed in overnight culture, and supernatants are tested for the presence of soluble factors which are able to affect human phagocytic cells in vitro.

Normal cells are also assayed for their ability to produce this factor. IL-2 dependent T cell lines have been developed to further characterize the source of lymphokine activity termed PIF or phagocytosis inducing factor. Preliminary biochemical characterization of PIF has been conducted.

GPO 914-918

Project Description

Objectives:

To determine whether certain human malignant lymphomas (especially those of peripheral T-cell origin) produce a factor (or factors) which can stimulate the phagocytic ability of human macrophages.

Methods Employed:

Neoplastic cells are placed in tissue culture and 24 hour supernatants tested for their ability to induce an increase in the number of Fc receptors and to enhance the phagocytic activity of the human promyelocytic cell line HL60, the macrophage-like cell line U937, and normal peripheral blood monocytes. Fc receptors are assayed by measuring the specific Fc receptor dependent binding of ^{125}I-IgG. Phagocytosis is assayed using IgG coated OX-RBC or 1.5 μ fluorescent beads.

Major Findings:

Results indicate that occasional peripheral T-cell lymphomas may secrete a factor which stimulates the phagocytosis of antibody-coated RBC but not beads. This effect is independent of an increase in the number of Fc receptors on the macrophage cell surface. Normal peripheral blood lymphocytes stimulated in an allogeneic mixed leucocyte reaction or by CON-A elaborate a factor which induces phagocytosis and with CON-A stimulated lymphocytes, this effect is not associated with an increase in Fc receptors. However, Fc receptors appear to be required. Phagocytosis of IgG-coated particles but not IgM coated particles is induced. Furthermore, free IgG will inhibit phagocytosis but not free IgM. Further studies were undertaken to investigate other effects of CON-A and tumor cell supernatants on U937 cells. There is induction of lysosomal enzymes as well as certain normal differentiation antigens seen on mononuclear phagocytes.

Further attempts to define the clinicopathologic characteristics of the patients which elaborate this factor have revealed that it is associated almost exclusively with angiocentric immunoproliferative lesions, i.e. lymphomatoid granulomatosis in both its benign and malignant phases. Other mature T-cell malignancies including HTLV-positive and HTLV-negative cases have been negative.

Preliminary attempts to characterize the factor (phagocytosis inducing factor or PIF) have indicated that it is temperature sensitive unlike MAF. PIF activity is stable at pH 2.0 unlike γ-interferon. Moreover, purified γ-interferon, even in high concentrations was unable to reproduce this phenomenon. Similarly, monoclonal antibodies against γ-interferon had a minimal or absent inhibitory effect.

PIF activity can be derived from T4 positive T-cell clones but not T8 positive clones. It has an apparent molecular weight of 45,000 - 55,000 daltons.

Significance to Biomedical Research and the Program of the Institute:

Some patients with peripheral T-cell lymphomas develop a syndrome resembling malignant histiocytosis characterized by fever, hepatosplenomegaly, and pancytopenia associated with histiocytosis and marked erythrophagocytosis within the reticuloendothelial system. The demonstration that neoplastic T cells from such a patient can secrete a factor capable of stimulating macrophage in vitro gives some insight into the pathogenesis of this syndrome, and also contributes to a greater understanding of the nature of normal lymphocyte/macrophage interaction.

The exclusive association of PIF activity with the angiocentric immunoproliferative lesion but not other peripheral T-cell neoplasms confirms that this is a distinctive clinicopathologic entity. Moreover, patients in both the "benign" and "malignant" phases of their disease show comparable activity. This observation would suggest that one is dealing with a single nosological entity and that true conversion to malignant lymphoma does not occur.

Hemophagocytic syndromes have been reported in other clinical settings, usually in association with an underlying immunodeficiency. In fact, the syndrome known as histiocytic medullary reticulosis appears to represent a hemophagocytic syndrome and not a true histiocytic malignancy. It is likely that PIF and perhaps other lymphokines as well are responsible for the pathogenesis of the hemophagocytic syndromes in general.

Proposed Course of Research:

Further studies are underway to define the characteristics of the angiocentric immunoproliferative lesions. Phenotypic studies are being pursued for their ability to distinguish benign and malignant phases of the disease. Clinicopathologic studies are being conducted to see if subclassification has clinical and prognostic relevance.

This project is being terminated as an independent study. Future work will be incorporated under Z01 CB 00550-05 LP.

Publications:

Jaffe, E.S.: Pathologic and clinical spectrum of post-thymic T-cell malignancies. Cancer Invest. 2: 413-426, 1984.

Simrell, C.R., Margolick, J.B., Crabtree, G.R., Cossman, J., Fauci, A.S., and Jaffe, E.S.: Lymphokine induced phagocytosis in angiocentric immunoproliferative lesions (AIL) and malignant lymphoma arising in AIL. Blood (in press)

Jaffe, E.S.: Malignant histiocytosis and true histiocytic lymphomas. Chapter 15. In Jaffe, E.S. (Ed.): Surgical Pathology of Lymph Nodes and Related Organs. Series: Major Problems in Pathology. Philadelphia, W.B. Saunders, 1985, pp. 381-411.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00552-05 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular basis of the diagnosis of human lymphoproliferative disease

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principel Investigator.) (Name, title, laboratory, end institute affiliation)*

PI:	J. Cossman	Senior Investigator	LP, NCI
OTHER:	M. Raffeld	Staff Fellow	LP, NCI
	E. Lipford	Expert	LP, NCI
	J. Sundeen	Guest Worker	LP, NCI
	L. Neckers	Expert	LP, NCI
	S. Pittaluga	Visiting Fellow	LP, NCI

COOPERATING UNITS *(if any)*

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Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2 1/2	1/2

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have undertaken a series of investigations aimed at improving the precision of diagnosis and classification of human lymphoproliferative disease. Our approach is based on determination of lineage and differentiation through the use of monoclonal antibodies, flow cytometry and molecular genetics. As a result of these studies, we have demonstrated a phenotypic definition of clinically relevant classes of lymphoma and have identified cellular marker combinations that distinguish between benign and malignant processes.

Monoclonality was found in nearly all malignant lymphoid neoplasms by Southern blot hybridization by the detection of gene rearrangements. However, we have discovered that monoclonality itself is not pathognomonic of malignancy since some types of nonmalignant lymphoid proliferations consistently exhibit monoclonality. In other studies concerning cellular lineage, DNA from patient biopsies tested for T-cell receptor gene rearrangements revealed a lack of rearrangement in nearly all B-cell neoplasms, clonal rearrangement in angioimmunoblastic lymphadenopathy (AILD), Tγ lymphoproliferative disorder and mature T-cell neoplasms, and a hierarchy of surface molecule expression and gene rearrangement in neoplasms of T-cell precursors.

Despite advances in the cellular origins of non-Hodgkin's lymphoproliferative disease, the lineage and clonal derivation of Hodgkin's disease remains largely unknown. Studies have been hampered by the lack of purified malignant cells from Hodgkin's tissues. We have prepared highly enriched Hodgkin's cell suspension from several patients and are analyzing them for DNA rearrangements, viral genomes and expression of mRNA and protein.

Project Description

Objectives:

To accurately identify and diagnose lymphoproliferative diseases and determine their cellular lineage and derivation.

Methods Employed:

Flow cytometry, DNA and RNA hybridization, tissue culture, density gradient centrifugation

Major Findings:

1. Phenotypic analysis discriminates among the major classes of indolent B-cell neoplasms by differential expression of developmentally regulated cell surface molecules.

2. Nearly all human B- and T-lymphoid malignant neoplasms are monoclonal.

3. Occult lymphoma is detectable in patient tissues which lack evidence of disease by histology or immunological studies.

4. T- and B-cell lineage of diffuse aggressive lymphomas, as determined by phenotypic studies, has been confirmed by detection of appropriately rearranged T-cell receptor or immunoglobulin genes.

5. Molecular genetic analysis has identified a subclass of diffuse, aggressive lymphomas that contains the t(14;18) chromosomal translocation typical of low-grade follicular lymphoma.

6. Angioimmunoblastic lymphadenopathy (AILD), in all cases tested, was monoclonal and contained either rearrangements of immunoglobulin genes, T-cell receptor genes, or both, simultaneously.

7. Monoclonality and T-cell lineage were demonstrated by rearrangement of the T-cell receptor gene in Tγ-lymphoproliferative disorder.

8. Benign lymphoid hyperplasias, other than those indicated above, showed no evidence of monoclonality. Included are lymphadenopathy, lymphocytosis and chronic infectious mononucleosis.

9. Precursor T-cell neoplasms occupy sequential differentiation compartments during early T-cell development as shown by the coordinated expression of surface membrane molecules and T-cell receptor rearrangements.

10. Reed-Sternberg cells and their variants can be isolated from tissues involved by Hodgkin's disease. Enrichment produces a sufficient frequency of Reed-Sternberg cells and variants to enable detection of their DNA and RNA sequences by molecular hybridization.

Significance to Biomedical Research and the Program of the Institute:

Human lymphoproliferative disease encompasses a broad range of clinical and biological entities. Development of a biological basis for their diagnosis and classification provides for appropriate selection of therapy. Moreover, human lymphoid neplasms serve as models for the investigation of lymphocyte differentiation, cellular lineage and clonal expansion. Our findings have helped establish biological techniques, i.e., monoclonal antibodies and molecular genetics, as tools for diagnostic hematopathology.

Proposed Course of Research:

1. Relate characteristic phenotypes of lymphoproliferative clinicopathological processes to gene rearrangement, chromosomal translocation and monoclonality.

2. Firmly establish a baseline for the frequency of monoclonality in lymphoid hyperplasia.

3. Probe histiocytic proliferations for rearrangement and expression of immunoglobulin genes and proto-oncogenes known to be activated in monocytes.

4. Examine the clonality and lineage of Hodgkin's disease by searching for gene rearrangement and expression.

5. Determine the in vitro inducibility of differentiation in precursor T-cell neoplasms. Cases in which the T-cell receptor genes are rearranged but not expressed will be tested for T-cell receptor transcription and expression of T3 molecular complex. Relate the acquisition of cell surface proteins to a hierarchy of T-cell receptor subunit gene rearrangements and expression.

6. The detection of occult disease in patients with low-grade lymphomas in apparent remission will be tested for by DNA hybridization and FACS analysis.

Publications:

Cossman, J., Jaffe, E.S., and Fisher, R.I.: Immunologic phenotypes of diffuse, aggressive non-Hodgkin's lymphomas. Correlation with clinical features. Cancer 54: 1310-1317, 1984.

Longo, D., Gelmann, E.P., Cossman, J., Young, R.A., Gallo, R.C., and Matis, L.A.: The isolation of a human T-cell leukemia/lymphoma virus (HTLV) transformed B lymphocyte clone from a patient with HTLV-associated adult T-cell leukemia. Nature 310: 505-506, 1984.

Mitsuya, H., Cossman, J., Guo, H.-G., Megson, O.J., Kao, C.S., Reitz, M.S., and Broder, S.: Clonal analysis of a long-term immune T-cell line reactive against human T-cell leukemia/lymphoma virus (HTLV). Science 225: 1484-1486, 1984.

Hecht, T., Longo, D.L., Cossman, J., Bolen, J.B., Hsu, S.-M., Israel, M., and Fisher, R.I.: Production of monoclonal antibody that selectively binds Reed-Sternberg cells. J. Immunol. (in press)

Fisher, R.I., Cossman, J., Diehl, V., and Volkman, D.J.: Antigen presentation by Hodgkin's disease cells. J. Clin. Invest. (in press)

Tsujimoto, Y., Jaffe, E., Cossman, J., Gorham, J., Nowell, P.C., and Croce, C.M.: Clustering of breakpoints on chromosome 11 in human B-cell neoplasms with the t(11;14) chromosome translocation. Nature (in press)

Kessler, D.J., Heilman, C.A., Cossman, J., Maguire, R.T., and Thorgeirsson, S.S.: Transformation of EBV immortalized human B cells by chemical carcinogens. Cancer Res. (in press)

Cossman, J., Bakhshi, A., and Korsmeyer, S.J.: Immunoglobulin gene rearrangements in the diagnosis of lymphoid neoplasms. In Manual of Clinical Laboratory Immunology (3rd edition), American Society for Microbiology, Washington, D.C. (in press)

Nagai, H., Fisher, R.I., Cossman, J., and Oppenheim, J.J.: Decreased expression of monocyte DR antigens in patients with Hodgkin's disease. J. Immunol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00850-03 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Clonal evolution of lymphoid neoplasms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Cossman	Senior Investigator	LP, NCI
OTHER:	M. Raffeld	Medical Staff Fellow	LP, NCI
	S. Pittaluga	Visiting Fellow	LP, NCI
	M. Uppenkamp	Guest Researcher	LP, NCI
	E. Lipford	Expert	LP, NCI

COOPERATING UNITS *(if any)*

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SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2 1/2	2 1/4	1/4

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously demonstrated that human B lymphoid leukemias and lymphomas are monoclonal neoplasms which, although arrested during development, retain a finite capacity to differentiate when appropriately activated. These investigations allowed us to uncover the natural clonal evolution of follicular lymphoma in vivo by the use of anti-idiotype antibody. We are now examining the frequency of occurrence and mechanism of tumor clonal evolution in human lymphoid neoplasms.

Multiple samples from 30 patients were surveyed for clonal fidelity by Southern blot analysis of rearranged immunoglobulin or T-cell receptor genes. Evidence for a single progenitor cell was established in each case studied since at least one gene rearrangement was held in common by independent samples from each individual. In most cases, all rearranged loci were retained in the same pattern throughout the course of disease. By contrast, we discovered a remarkably high frequency (35%) of clonal evolution among follicular B-cell lymphomas. Mechanisms of clonal evolution may include new variable region gene rearrangements, heavy chain constant region isotype switching and altered (mutated?) idiotype. Deduced genealogies of the related clones argue for an immature follicular lymphoma progenitor cell in which some immunoglobulin genes still remain in a germline configuration. Our finding of conservation of the immunoglobulin allele involved in a t(14;18) translocation, despite new rearrangements of the opposite productive allele, implicates chromosomal rearrangement as an early event in progenitor cell development.

GPO 914-918

Project Description

Objectives:

To investigate clonal evolution of lymphoid neoplasms with respect to 1) the characterization of the genetic events resulting in altered immunoglobulin genes, 2) the impact of new idiotypes on escape from host immune recognition and 3) the correlation of clonal evolution and tumor progression.

Methods Employed:

Genomic DNA (Southern) hybridization, RNA (Northern) blots, cell culture, cytotoxicity, ELISA, monoclonal antibody immunostaining, flow cytometry.

Major Findings:

1. Anti-idiotype antibody uncovered the rapid emergence of a subclone of follicular lymphoma shortly after spontaneous tumor regression. Alteration of idiotype was likely attributable to somatic mutation of a V-region gene.

2. High frequency (35%) of clonal evolution in follicular lymphoma resulted from new rearrangements of immunoglobulin genes.

3. Conservation of the translocated chromosome 18 element despite clonal evolution of follicular B-cell lymphomas.

Significance to Biomedical Research and the Program of the Institute:

Indolent B-cell lymphomas remain largely incurable even though a clinical complete remission can be achieved in most patients with chemotherapy. However, relapse usually occurs with recurrence of a lymphoma of either the original clinicopathological type or of a more aggressive type. Our results demonstrate that recurrence is not due to emergence of a second tumor but rather regrowth of the initial lymphoma and, therefore, in such instances therapy was ineffective at eradicating the tumor in its entirety. Moreover, frequent clonal evolution points to an occult immature progenitor cell as the source of follicular lymphoma. Finally, the capacity of follicular lymphomas to frequently alter their surface membrane idiotype may allow them to escape detection by a host immune response and may account for the well documented waxing and waning during the natural course of this disease. Alternative means of therapy are being sought for follicular lymphoma patients and future clinical interventions could exploit such an immune response.

Proposed Course of Research:

1. Evidence for V-region joinings, CH isotype switching or somatic mutation as a basis for clonal evolution will be investigated. DNA from those lymphomas lacking t(14;18) will be surveyed for translocations of other chromosomal elements.

2. Evidence of clonal evolution will be analyzed in DNA obtained from peripheral blood and bone marrow lymphocytes in patients with low-grade lymphoma.

3. In vitro models of clonal evolution in follicular B-cell neoplasms will be explored by assaying induced subclones for alteration of immunoglobulin genes and their translation products.

4. To assess the effect of altered idiotype on immune recognition, we will attempt to derive T-cell clones from involved lymph nodes and test them for cytotoxicity and suppression against variant subclone B-cell targets.

5. T-cell neoplasms will be further analyzed for clonal evolution by searching for new rearrangements of T-cell receptor genes.

Publications:

Raffeld, M., Neckers, L.M., Longo, D., and Cossman, J.: Spontaneous alteration of idiotype in a monoclonal B-cell lymphoma: Escape from detection by anti-idiotype. N. Engl. J. Med. (in press)

Braziel, R.M., Sussman, E., Jaffe, E.S., Neckers, L.M., and Cossman, J.: Induction of immunoglobulin secretion in follicular non-Hodgkin's lymphomas: Role of immunoregulatory T cells. Blood (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00851-03 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanism of TPA-induced immunoglobulin secretion by CLL cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.M. Neckers	Expert	LP, NCI
OTHER:	S. Pittaluga	Fogarty Fellow	LP, NCI
	J. Cossman	Sr. Assistant Surgeon	LP, NCI
	J.B. Trepel	Biologist	LP, NCI
	R.C. McGlennen	Medical Student	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have demonstrated that the phorbol ester TPA is capable of causing induction of immunoglobulin synthesis in chronic lymphocytic leukemia cells (CLL). This induction involves increased levels of mRNA coding for the secretory form of IgM. Our goal in this study is to discern the mechanism(s) whereby TPA exerts its effects on these CLL cells. We have found that TPA induces IgM mRNA accumulation in a calcium-independent manner, but that TPA-induced secretion is calcium-dependent. Furthermore in normal B cells, induction of IgM mRNA by mitogen is calcium-independent while T cell supernatant stimulated IgM secretion is calcium dependent. Thus, TPA can mimic both mitogen and T cell factors in their effects on B cell activation.

We have also identified a monoclonal antibody capable of inhibiting both TPA-induced, T cell supernatant-induced and constitutive IgM secretion. This antibody may recognize a membrane protein which is phosphorylated during the course of IgM secretion.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To study the mechanism(s) by which TPA induces immunoglobulin secretion in normal as well as malignant B cells.

Methods Employed:

Use of ELISA assays to measure Ig and various methods to measure cellular mRNA for Ig gene expression are used in addition to those already listed.

Major Findings:

TPA induces Ig secretion in normal and malignant B cells by two distinct mechanisms which can be distinguished by their calcium dependence. mRNA induction does not require extracellular calcium while Ig secretion does. In its effects, TPA mimics both antigen (mRNA induction) and a T cell lymphokine (Ig secretion). We have also identified an antibody which blocks TPA-stimulated as well as constitutive IgM secretion. The membrane protein it recognizes may be phosphorylated during the course of IgM secretion.

Significance to Biomedical Research and the Program of the Institute:

B-cell lymphomas and leukemias are thought to be models for normal B cells arrested at different stages of development. The plasma cell stage of B-cell differentiation, when B cells secrete immunoglobulin, is thought to be the end stage for these cells. If we can determine how CLL cells, B cells arrested at an earlier stage of development, can be made to progress to end stage differentiation by TPA, we will have a better understanding of the regulatory processes of normal B-cell differentiation as well as what goes wrong in CLL cells to prohibit them from fully differentiating.

Proposed Course of Research:

Currently, due to a staff shortage, we have no plans to continue this project during the next fiscal year.

Publications:

Pittaluga, S., Cossman, J., Trepel, J.B., and Neckers, L.M.: Inhibition of immunoglobulin secretion, but not immunoglobulin synthesis, by a monoclonal antibody. In Proceedings of 2nd International Workshop on Leukocyte Typing. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00855-03 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pathologic features of HTLV-associated diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

PI:	E.S. Jaffe	Chief, Hematopathology Section	LP, NCI
OTHER:	W.A. Blattner	Senior Investigator	EEB, NCI
	R.C. Gallo	Senior Investigator	LTCB, NCI

COOPERATING UNITS *(If any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Pathologic material from patients identified to be seropositive for HTLV-I is reviewed and correlated with clinical and epidemiologic features of disease. Material is derived from patients in the United States as well as other parts of the world. Where possible, immunologic phenotyping of the lymphomas is performed and tumor DNA is directly analyzed for viral genome.

In selected populations where HTLV-I is endemic, such as Jamaica, prospective studies of all newly diagnosed lymphoma patients are conducted. Such studies are useful in identifying the clinicopathologic spectrum of HTLV-I associated diseases.

PHS 6040 (Rev. 1/84) GPO 914-916

Project Description

Objectives:

To determine if HTLV-I, a human T-lymphotropic retrovirus, is associated with a distinct clinicopathologic entity and to determine if pathologic criteria can be developed to recognize this entity.

Methods Employed:

Screening of sera for antibodies to the viral core proteins p19 and p24. Isolation of virus from tumor cells. Pathologic review of all positively identified samples.

Major Findings:

HTLV-I is associated with a distinct clinicopathologic syndrome. Although there is some overlap with other mature T-cell malignancies, some unique features have been identified. These include a leukemic pattern of lymph node infiltration and a broad spectrum of cytologic types. Although the classical syndrome of adult T-cell leukemia is a fulminant disease poorly responsive to chemotherapy, chronic forms of HTLV-associated disease have been more recently identified. So-called smoldering ATL is associated with chronic cutaneous manifestations, circulating atypical cells and an absence of hypercalcemia, hepatosplenomegaly and significant lymphadenopathy.

Significance to Biomedical Research and the Program of the Institute:

The recognition of defined clinicopathologic criteria for the identification of HTLV-I-associated malignancies should aid in diagnosis and further definition of the epidemiology of this disease.

Proposed Course of Research:

Analysis of prospective series of lymphomas in parts of the world endemic for HTLV-I.

Publications:

Blattner, W.A., Blayney, D.W., Jaffe, E.S., Robert-Guroff, M., Kalyanaraman, V.S., and Gallo, R.C.: Epidemiology of HTLV-associated leukemia. In Neth, Gallo, Greaves, Moore, and Winkler (Eds.): Modern Trends in Human Leukemia V, Vol. 28: Haematology and Blood Transfusion. Berlin/Heidelberg, Springer-Verlag, 1983, pp. 148-155.

Blattner, W.A., Clark, J.W., Gibbs, W.N., Jaffe, E.S., Robert-Guroff, M., Saxinger, C., and Gallo, R.C.: HTLV: Epidemiology and relationship to human malignancy. In Gallo, R.C. and Essex, M. (Eds.): Cancer Cells, Human T-Cell Leukemia/Lymphoma Viruses, Vol. 3. New York, Cold Spring Harbor Press, 1984, pp. 267-274.

Whang-Peng, J., Bunn, P.A., Knutsen, T., Kao-Shan, C.S., Broder, S., Jaffe, E.S., Gelmann, E., Blattner, W., Lofters, W., Young, R.C., and Gallo, R.C.: cytogenetic studies in human T-cell lymphoma virus (HTLV)-positive leukemia-lymphoma in the USA. J. Natl. Cancer Inst. 74: 357-369, 1985.

Jaffe, E.S., Clark, J., Steis, R., Blattner, W., Macher, A.M., Longo, D.L., and Reichert, C.M.: Lymph node pathology of HTLV and HTLV-associated neoplasms. Cancer Res. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00864-03 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Control of the interleukin 2 gene in normal and malignant cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N.J. Holbrook	Senior Staff Fellow	LP, NCI
OTHER:	G.R. Crabtree (from Oct. 1, 1984- Dec. 30, 1984)	Medical Officer	LP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Interleukin 2 (IL-2), or T-cell growth factor, is a 15,000 dalton polypeptide in humans which is believed to be responsible for the clonal expansion of normal T lymphocytes during the immune response. Transcription of this small protein is inducible by mitogens such as phytohemagglutinin or concanavalin A in normal human lymphocytes. Recent evidence indicates that it may control the replication of certain human malignant T cells. It has been found that production of IL-2 is completely inhibited by glucocorticoids and cyclosporin A, both of which are powerful immunosuppressive agents. Both glucocorticoids and cyclosporin A inhibit IL-2 production at the level of transcription.

The goals of our studies will be to define the factors controlling expression of the IL-2 gene in normal and malignant cells and to attempt to understand the mechanisms through which these factors exert their effects. To this end we have cloned and completely sequenced the human gene for IL-2. We have been making various hybrid gene constructions including linking the IL-2 promoter and 5' sequences to the chloramphenicol acetyl transferase (CAT) structural gene and placing the IL-2 cDNA under the control of the mouse metallothionein or SV40 viral promoters. We have begun to transfect these constructs into various cell types.

In collaboration with Yuan DeVries, we have recently found that the IL-2 gene in MLA 144 cells, a gibbon ape T-cell line, is rearranged. The rearrangement is due, at least in part, to the insertion of the gibbon ape leukemia virus (GALV) long terminal repeat (LTR) in the gene. We are attempting to identify the role this viral LTR plays in controlling IL-2 expression in these cells.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

1) To understand the regulation of the human IL-2 gene by mitogens and glucocorticoids at the genetic level. 2) To investigate if and how this gene is involved in certain human T-cell lymphomas and leukemias.

Methods Employed:

Using a chemically synthesized oligonucleotide probe, the cDNA for IL-2 was cloned from a cDNA library made from a human IL-2 producing cell line (Jurkat). The complete gene was subsequently isolated by screening genomic libraries from lymphocytes and its structure determined by sequencing the DNA. DNA was purified from normal and malignant cells and examined by Southern blotting. Using standard cloning procedures and eukaryotic expression, vectors were used to make various fusion-gene constructions. Their expression is being tested in transient and stable transfection systems using $CaPO_4$, DEAE-dextran, electroporation and protoplast fusion procedures.

Major Findings:

We have stably transfected monkey and mouse fibroblasts with fusion genes containing the IL-2 cDNA expressed under the control of the mouse metallothionein and SV40 viral promoters. IL-2 is produced in these stable transformants.

In collaboration with Yuan DeVries (E.I. duPont, Glenolden, PA), we have found that in MLA 144 cells, a gibbon ape T-cell line that constitutively produces IL-2, the gene is rearranged. At least part of the rearrangement is due to an insertion of the long terminal repeat (LTR) of the gibbon ape leukemia virus (GALV) at the 3' end of the gene.

We have shown that IL-2 mRNA can be induced *in vivo* in the monkey following antigenic stimulation with Freund's complete adjuvant. We have found that sequences in the 5' flanking region of the IL-2 gene share limited homology to HTLV1 which, like IL-2, is T-cell-tropic.

Significance to Biomedical Research and the Program of the Institute:

Understanding how the IL-2 gene is normally regulated by mitogens or antigens and glucocorticoids will provide insight into what factors are responsible for its altered regulation in aberrant cell growth. In the case of the MLA 144 cells, the role that the viral LTR insertion into the IL-2 gene plays in the malignancy of these cells can directly be addressed. This provides a useful model for understanding the pathogenesis of human retroviruses.

Proposed Course of Research:

With hybrid constructions linking the 5' regulatory sequences of IL-2 to the CAT structural gene, we will attempt to locate what sequences are responsible for mitogen stimulation of IL-2 expression. We will determine whether glucocorticoids interact directly with the IL-2 gene to inhibit its expression or indirectly via another gene product.

We will examine the role the GALV LTR plays in controlling expression of the IL-2 gene in MLA 144 cells. We will attempt to relate the enhanced expression of the IL-2 gene in these as well as certain human malignancies to the uncontrolled growth of these cells.

Publications:

Holbrook, N.J., Smith, K.A., Fornace, A.J., Jr., Comeau, C.M., Wiskocil, R.L., and Crabtree, G.R.: Complete sequence of the gene for T-cell growth factor and its structure in normal and malignant cells. Proc. Natl. Acad. Sci. USA 81: 1634-1638, 1984.

Holbrook, N.J., Lieber, M., and Crabtree, G.R.: DNA sequence of the 5' flanking region of the human interleukin 2 gene: homologies with adult T-cell leukemia virus. Nucl. Acids Res. 12: 5005-5013, 1984.

Chen, S.J., Holbrook, N.J., Mitchell, K.F., Vallone, C.A., Crabtree, G.R., and Lin, Y.: A viral long terminal repeat in the IL-2 gene of a cell line which constitutively produces IL-2. Proc. Natl. Acad. Sci. USA (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00881-04 LP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Regulation of cell growth by transferrin receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L.M. Neckers	Expert	LP, NCI
OTHER:	J.B. Trepel	Biologist	LP, NCI
	S. Bauer	Graduate Student	LG, NCI
	R. Nordan	Staff Fellow	LG, NCI
	O. Colamonici	Fogarty Fellow	LP, NCI

COOPERATING UNITS *(if any)*

Laboratory of Genetics, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

All cells studied to date require transferrin for growth. We and others have shown that antibodies to the transferrin receptor block the growth of lymphoblastoid cell lines. In mitogen-stimulated lymphocytes, these antibodies block proliferation. We are studying the processes which regulate the appearance of these receptors in lymphocytes and lymphoblastoid cell lines, and the function of these receptors in cell growth and metabolism. We have demonstrated that G_1 arrest occurs in both normal and malignant cells when transferrin receptors are blocked, even if cells are expressing high levels of growth factor receptor messenger RNA and c-myc and c-myb messenger RNA. Furthermore, either blockade of calcium channels or addition of cAMP to cells results in G_1 arrest and loss of transferrin receptor mRNA. The effect of cAMP can be detected at the level of transcription.

Project Description

Objectives:

To discern the regulation of transferrin receptor appearance in mitogen-stimulated peripheral blood lymphocytes as well as transformed cell lines, and to study the role of these receptors in proliferation and growth.

Methods Employed:

Quantitation of transferrin receptors is made by use of the cell sorter. DNA, RNA and protein synthesis are measured by incorporation by cell of radioactive substrates. Cellular DNA and RNA are studied using various molecular probes following standard techniques.

Major Findings:

1. Normal cells regulate transferrin receptor appearance by carefully controlled tissue-specific growth factors and their receptors. This regulation is lost in malignant cells and transferrin receptor expression becomes constitutive.

2. Nevertheless, both growth factor-dependent and constitutive transferrin expression is calcium dependent, while transcription of the IL-2 receptor gene and genes for c-myc and c-myb are not calcium dependent.

3. Transferrin receptor redistribution and its associated hyperphosphorylation can be dissociated by blockade of calcium channels. Hyperphosphorylation is not calcium dependent, but redistribution is.

4. Redistribution of transferrin receptors may reduce surface receptor levels by 60% without affecting proliferation.

5. Cyclic AMP terminates transferrin receptor gene transcription in normal and some malignant cells. This is correlated with a G_1 arrest.

6. Growth factor dependent mouse plasmacytoma cell transferrin receptor synthesis and mRNA expression depends on continuous presence of growth factor. Removal of growth factor results in loss of receptor mRNA and G_1 arrest.

Significance to Biomedical Research and the Program of the Institute:

What triggers a cell to divide is currently under intense investigation. Learning in a normal cell what this trigger(s) is(are), would be a monumental step in the understanding of cancer. Since the transferrin receptor is present on every type of dividing cell studied to date, and since its removal prevents cell division, we feel that studying the regulation of this receptor in normal and transformed lymphocytes will add to the understanding of what regulates cell division.

Proposed Course of Research:

We plan to continue our studies in two basic areas: 1) the role of the transferrin receptor in cell growth (using transformed cell lines) and 2) the role of the receptor in activation, proliferation and differentiation of normal lymphoid cells. We are currently investigating the relationship of transferrin receptor expression to various oncogenes and their products to determine if oncogene activation is needed for transferrin receptor expression in normal and malignant cells. This is in collaboration with Drs. M. Potter and F. Muchinsky of NCI. We are also focusing on regulation of transferrin receptor gene transcription using a full length cDNA probe.

Publications:

Neckers, L.M. and Cossman, J.: Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin-2 (TCGF). In Goldstein, A.L. (Ed.): Thymic Hormones and Lymphokines. New York, Plenum Publ. Corp., 1984, pp. 383-394.

Neckers, L.M., Yenokida, G., and James, S.P.: The role of the transferrin receptor in human B lymphocyte activation. J. Immunol. 133: 2437-2441, 1984.

Neckers, L.M.: Transferrin receptor regulation of proliferation in normal and neoplastic B cells. In Potter, M., Melchers, F., and Wiegert, H. (Eds.): Current Topics in Microbiology and Immunology 113: 62-68, 1985.

Neckers, L.M., Yenokida, G., and James, S.P.: Transferrin receptors are required for B cell proliferation but not for immunoglobulin secretion. J. Cell. Biochem. 27: 377-389, 1985.

Trepel, J.B., Colamonici, O.R., Klausner, R.D., and Neckers, L.M.: Cyclic nucleotide regulation of promyelocytic cell transferrin receptor distribution and synthesis. Short Report - Proceedings of the 1985 Miami Winter Symposium, 1985.

Trepel, J.B., Klausner, R.D., Colamonici, O.R., Pittaluga, S., and Neckers, L.M.: Down-regulation of promyelocytic cell transferrin receptor expression by cholera toxin and cyclic adenosine monophosphate. Human Leukocyte Differentiation Antigens II. (in press)

Neckers, L.M., Bauer, S., and Norden, R.: Growth factor regulation of transferrin receptor synthesis and expression in mouse plasmacytomas. In Melchers, F. and Potter, M. (Eds.): Mechanisms of B Cell Neoplasia. (in press)

SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY REPORT
DCBD, NCI
October 1, 1984 through September 30, 1985

The Dermatology Branch conducts both clinical and basic research studying the etiology, diagnosis and treatment of inflammatory and malignant diseases involving the skin and the host's response to these diseases. The basic research involves biochemical as well as biological studies of skin and is subdivided into six separate, though frequently interacting, areas. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center (approximately 1500 patients are seen in consultation each year). The main research achievements of the Dermatology Branch for the past year are as follows:

Immunopathologic Mechanisms Involved in Inflammatory and Neoplastic Skin Diseases:

A major focus of our studies of the immunopathology of skin diseases concerns the effects of ultraviolet radiation (UV) on epidermal immune responses. Ultraviolet light irradiation modulates the antigen presenting function of Langerhans cells in such a way so that UV irradiated epidermal cells stimulate allogeneic T cells poorly if used immediately after irradiation and stimulate vigorously if taken 3 days after irradiation. We have found that after UV an $OKT6^{-}DR^{+}$ melanophagic cell appears in the epidermis and is responsible for the enhanced stimulation. We have now characterized this cell further - it is $OKM5^{+}$ $OKM1^{-}$ and appears analogous functionally to the $OKM5^{+}$ $OKM1^{-}$ peripheral blood cells. This cell is derived from bone marrow as it is T 200 and HLe 1 positive. These findings in humans are in concert with studies in mice which indicate the appearance of a potent accessory cell 3 days after UV. To further our studies of the UV effect on antigen presenting cells we have found that UV inhibits the ability of Langerhans cells and various other antigen presenting cells to process various types of antigens. This inhibition may be responsible for the ultimate generation of suppressor cells when antigen is seen in the context of an UV-treated antigen presenting cell. We have also demonstrated that in certain types of immunologic reactions in the skin, namely in graft versus host disease (in mice) keratinocytes can be induced to synthesize Ia antigens. The function of this Ia antigen is currently being sought. Another skin cell which is probably immunologically important is the recently identified dendritic Thy 1 positive murine epidermal cell. We have had some success at culturing a $Thy\ 1^{+}$ cell from mouse skin. Other studies suggest that the cell is not present in human skin.

Molecular Basis of Autoimmune Skin Diseases:

This laboratory was recently established to study autoantibody-mediated skin diseases in order to further our understanding not only of the pathophysiology of these diseases but also of the structure and function of normal epidermis and epidermal basement membrane zone (BMZ). Specifically, antibodies in these diseases define molecules in the normal epidermis. We have characterized the antigens defined by three of these diseases: bullous pemphigoid (BP), pemphigus vulgaris (PV), and pemphigus foliaceus (PF). We have defined the cells which synthesize these antigens, as well as the antigen defined by the autoantibodies

found in patients with epidermolysis bullosa acquisita (EBA). We have used the binding of antibodies to specific molecules to make diagnoses of EBA or BP in various complicated cases of these diseases. We have demonstrated that PV and PF autoantibodies bind distinct molecules. All PV autoantibodies bind the same molecule. Almost half of PF autoantibodies bind to a 155 kd glycoprotein which we have shown to be identical to desmoglein I, a desmosomal core glycoprotein. We are currently studying the molecular basis of Fago Selvagem, a form of pemphigus foliaceus which is endermic in Brazil.

Detection and Analysis of Circulating Immune Complexes:

We have performed the first prospective indepth immunological and immunopathological study of serum sickness in man. We have defined the time course of the clinical, immunological and immunopathological findings in human serum sickness. We have described a hitherto unknown cutaneous sign in humans specific for serum sickness. Since immune complexes may activate the complement system and since the complement fragments C5a and C3a are thought to be important in the pathogenesis of the inflammatory response in cutaneous and systemic diseases, we have purified C5a and studied its in vivo and in vitro reactivity. Its in vivo role was assessed by the first in-depth analysis of the cutaneous reactivity of this complement fragment in man. We have also studied the ability of C5a and C3a to modulate cell surface receptors for immunoglobulin and complement on the surface of leukocytes. Increasing evidence indicates that human endothelial cells, under certain circumstances, can be induced to become immunologically competent. In order to evaluate the role endothelial cells in immune complex mediated vasculitis we have isolated human umbilical vein endothelial cells, grown them in cell culture, and examined them for the presence of immunologically relevant cell surface antigens and receptors before and after stimulation with soluble mediators of immunoregulation.

Therapy of Skin Cancer and Disorders of Keratinization:

We are continuing to evaluate safety and effectiveness of new oral and topical agents particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne. Oral etretinate was more effective and less toxic than isotretinoin in the treatment of the disorders of keratinization. One chronic toxicity, "retinoid hyperostosis," characterized by anterior spinal ligament calcification and osteophyte formation of vertebrae, has been observed in 80% of patients treated with long-term, high-dose isotretinoin. Sixty percent of acne patients with moderate doses of isotretinoin for 9 months also developed vertebral osteophytes. Five patients with xeroderma pigmentosum were entered into a cancer chemoprevention study using isotretinoin. Of 5 patients entered into a phase I fenretinide-cancer chemoprevention study for basal cell carcinoma, 3 were discontinued due to toxicity (drug reaction, night blindness). This drug has now been withdrawn from further clinical chemoprevention trial.

Studies of DNA Repair in Normal Human Cells from Patients with Xeroderma Pigmentosum and Neurodegenerative Disorders:

Cytogenetic studies are being conducted to determine if DNA-damaging agents induce abnormal numbers of chromosome aberrations in human cells which have a hypersensitivity to the lethal effects of such agents. The development of such

a cytogenetic test would make it possible to detect hypersensitivity to DNA-damaging agents within a few days, as opposed to the currently employed colony-forming test which requires two to three weeks. Cells from patients with ataxia telangiectasia, tuberous sclerosis, Alzheimer and other diseases are being studied. We have detected in ataxia telangiectasia heterozygote fibroblasts an abnormally high number of chromosomal abberations after G2-irradiation of the cells with X-rays.

Ribonuclease H is an enzyme which hydrolyzes hybrid DNA (DNA:RNA). Hybrid DNA is apparently required for the initiation of DNA replication for semi-conservative DNA synthesis. It is not known whether or not hybrid DNA and RNAase-H also play a role in repair replication of damaged DNA. In conjunction with Dr. R. Crouch, Laboratory of Molecular Genetics, NICHHD, we have demonstrated the presence of R-Nase-H in cultured human fibroblasts and lymphoblastoid lines. Studies are being conducted to determine if R-Nase-H is inducible by DNA-damaging agents, a positive finding of which would indicate a putative role of the enzyme in DNA repair.

With Drs. D.S. Bergtold and M.M. Dizdaroglu, of Dr. Michael Simic's Ionizing Radiation Competency Program, National Bureau of Standards, Gaithersburg, MD, we are attempting to detect and identify specific forms of damage to DNA by means of capillary gas chromatography combined with mass spectrometry. Using these techniques Dr. Dizdaroglu can successfully identify numerous types of damage in purified calf thymus DNA. In this joint venture between our laboratories, we are attempting to detect such damage (and its repair) in the DNA of human cells we treat with X-rays.

Chemistry, Structure, and Biosynthesis of Mammalian Epidermal Keratin Filaments:

We are continuing our studies of the physico-chemical structure of keratin and other intermediate type filaments. In collaboration with Dr. Roop (LCCTP, DCE, NCI), we have isolated and fully characterized two keratin genes: the mouse 59 kD and human 67kD. We will now further characterize several features of these. Firstly, enhancer sequences and/or other sequences involved in the expression of these genes in epidermis will be characterized. Secondly, we will set up a cosmid library of mouse and human genomic DNA in an attempt to determine how the keratin genes are physically placed within the genome-whether the keratin genes are linked, their chromosomal locations, etc. Thirdly, preliminary data suggests that the two keratin genes and possibly those of other Types may have evolved from a common ancestor. Further detailed analyses of the genes and comparisons with other intermediate genes are in progress to expose this hypothesis.

Analysis of the α-helical sequences of keratin subunits show that they all have a common secondary structure in that they form four extended tracts of coiled-coil α-helix with another keratin, and that these tracts are separated by α-helical but non-coiled-coil linkers. These α-helical regions form 2-chain coiled-coils. Detailed comparisons of the sequences of these coiled-coil tracts show that there are distinctly different types of sequences. Acidic keratins (mouse 59, 55, 50kD, human 57kD) form Type I sequences. Neutral-basic keratins (mouse 67, 60, human 67kD) form Type II sequences. We have also shown that the other intermediate filament proteins, vimentin, desmin and glial fibrillary acidic protein, form Type III sequences. The neurofilament proteins form Type IV sequences. In other experiments in collaboration with Drs. Parry and

Steven, it has been shown that in keratins, the 2-chain, coiled-coil molecule consists of one Type I protein and one Type II protein, that is, in a heterodimer. In contrast, the Type III and Type IV proteins all form homodimers. In all cases, the two chains of the coiled-coil molecule are in exact-axial register.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03657-11 D

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

S.I. Katz, Branch Chief, Dermatology Branch, NCI

COOPERATING UNITS *(if any)*

Dermatology Department, USUHS, Bethesda

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.0	5	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

My clinical and laboratory endeavors involve two major areas of immunodermatology. The first deals with studies of patients with various forms of vesiculobullous diseases. We have not only provided detailed clinicoimmunopathological correlations of several heretofore poorly defined diseases, i.e. dermatitis herpetiformis, acquired epidermolysis bullosa and herpes gestationis but we have characterized the antigens to which the antibodies in some of these diseases bind. My second and major area of study is the role of the epidermis as an immunological tissue. We have demonstrated that within normal epidermis Langerhans cells are the only cells which 1) synthesize and express Ia antigens, 2) can present both soluble antigens and haptens to sensitized T cells, 3) are capable of allogeneic T cell stimulation in a mixed epidermal-lymphocyte proliferation system, 4) can induce hapten and allogeneic cytotoxic T lymphocytes in vitro, and 5) are of a mesenchymal origin. We have also demonstrated that keratinocytes produce an Interleukin 1-like cytokine which may serve as a second signal in generating T cell responses. Ultraviolet radiation has been shown to modulate many of these functions.

PHS 6040 (Rev. 1/84)

GPO 914-918

Other Professional Personnel:

S. Shimada	Visiting Fellow	Derm NCI
W. Caughman	Medical Staff Fellow	Derm NCI
K.D. Cooper	Senior Staff Fellow	Derm NCI
W. Aberer	Guest Researcher	Derm NCI

Project Description

Objectives:

1) To investigate the mechanisms involved in the expression of certain immunologic skin diseases, namely pemphigus, bullous pemphigoid, dermatitis herpetiformis and herpes gestationis.

2) To determine the cell surface characteristics of the lymphocytes and epidermal cells involved in these diseases.

3) To determine the ultrastructure and ultrastructural localization of antibodies in blistering skin diseases.

4) To characterize the chemical constituents of the basement membrane zone.

5) To determine the functional capabilities of Ia-bearing epidermal cells (Langerhans cells) in mice, guinea pigs, and humans as these cells play an integral role in antigen presentation and in allogeneic T cell activation.

6) To identify substances produced by epidermal cells which modulate immunological responses.

7) To identify and characterize normal cell surface, basement membrane and cytoplasmic structures in human skin and to determine their possible role in skin cancer, wound healing, and in blistering diseases.

8) To characterize the origin and function of dendritic thy-1 positive cells in murine skin and to identify a human analogue.

9) To discover how ultraviolet radiation modulates epidermal immunological functions.

10) To identify the lesion induced by UV in antigen presenting cells.

11) To develop clones of T cells which have specificity for skin-related antigens.

Material:

Skin biopsies, either punch or Castroviejo keratome slices are used. Also the small intestine of patients with dermatitis herpetiformis are studied in order to determine their antigen binding characteristics. Blister fluid studies for inflammatory mediators are also under investigation. The roofs of suction blisters are also assessed for the distribution of Langerhans cells and production of immunomodulating factors. Guinea pigs, mice and rabbits are used for the identification of lymphocyte and epidermal cell surface antigens as well as for the production of antibodies. Mouse skin is used to prepare epidermal cell suspensions which are used for sensitization.

Methods Employed:

Direct and Indirect Immunofluorescence, Cell Mediated Cytotoxicity, Immuno chemical methods for identifying immunoglobulins and immune complexes. Radioimmunoassays. Mixed leukocyte cultures and *in vitro* antigen priming studies. Delayed type hypersensitivity reactions including contact hypersensitivity are generated by skin painting and by the injection of haptenated cells into syngeneic mice. Radioimmunoprecipitation techniques are also employed as are standard techniques for the production of monoclonal antibodies.

Major Findings:

1) In vivo studies indicate that Langerhans cells play an integral role in the induction of contact hypersensitivity. Epidermal cells devoid of Langerhans cells cannot perform this function. In vitro Langerhans cells have been shown to have strong stimulatory activity in TNP specific and allostimulatory proliferation and cytotoxic T lymphocyte systems. We have recently generated hapten specific T cell clones which respond preferentially to haptenated epidermal cells rather than to haptenated spleen cells suggesting that unique epitopes may be recognized on epidermal cells.

2) Ultraviolet light irradiation modulates the antigen presenting function of Langerhans cells in such a way so that UV irradiated epidermal cells stimulate allogeneic T cells poorly if used immediately after irradiation and stimulate vigorously if taken 3 days after irradiation. This occurs because of the appearance of a melanophagic $OKT6^{-}DR^{+}$ cell within the epidermis three days after UV. We have now functionally characterized this cell.

3) Keratinocytes devoid of Langerhans cells produce a factor which has Interleukin (IL) I activity; that is , it enhances PHA induced thymocyte proliferation. It has many of the same physicochemical characteristics of IL 1. We have termed this factor ETAF. ETAF serves to reconstitute UV induced abrogation of allogeneic T cell stimulation. It also induces fever (like endogenous pyrogen) and is chemotoctic for neutrophils. Other factors such as indomethacin are produced by keratinocytes and these also modulate immunological functions.

4) We have produced monoclonal antibodies against normal epidermal cell constituents and are currently characterizing the antigens and studying their role in various pathologic states, such as wound healing and skin tumor formation. One such antibody KF-1 identifies a structure in the basement membrane zone which is defective in patients with epidermolysis bullosa. The antigen which is identified by KF-1 appears ontogenetically at 16 weeks of fetal life.

5) We have demonstrated that in certain types of immunologic reactions in the skin, namely in graft versus host disease (in mice) keratinocytes can be induced to synthesize Ia antigens. The Ia antigens on keratinocytes do not induce allogeneic T cell proliferation. The function(s) of these Ia positive keratinocytes is currently under study.

6) We have begun to develop a system for culturing murine dendritic thy 1 positive epidermal cells. This will facilitate study of the function of these cells which we have demonstrated to be of bone marrow origin. We have exhaustively searched for the human analogue of this cell but have been unable to demonstrate such a cell.

Significance to Cancer Research:

A basic understanding of immunologic injury in various autoimmune disease states is important in interpreting and furthering current concepts in self-recognition. Pemphigus is associated with thymoma and myasthenia gravis and its study may provide a clue as to the association between pemphigus and other malignancies. Our in depth studies of herpes gestationis, an immunologic blistering disease of pregnancy, may provide important clues to maternal-fetal interactions. The role of Langerhans cells in immune reactions in in the skin and their possible function or dysfunction after ultraviolet light exposure should provide some insight into their role in skin tumor formation. The study of the basement membrane and its disruption by cancer cells is essential to the study of tumor invasion.

Proposed Course of Project:

Outlined above.

Publications:

Katz, S.I.: The role of Langerhans cells in allergic contact dermatitis. Am. J. Dermatopathology (in press).

Katz, S.I., Cooper, K.D., Iijima, M., Tsuchida, T.: The role of Langerhans cells in antigen presentation. Invest. Derm. (in press).

Katz, S.I.: The skin as an immunological organ. J. Amer. Acad. Dermatol. (in press).

Shimada, S., Katz, S.I.: TNP-specific Ly-2^+ cytolytic T cell clones preferentially respond to TNP-conjugated epidermal cells. J. Immunol. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03666-07 D

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

Chemical Mediators of Inflammation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboretory, and institute affiliation)*

Thomas J. Lawley, M.D., Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(it eny)*

LCI, NIAID, Clinical Hematology Branch, NHLBI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Antigen-antibody complexes play important roles in a variety of human systemic and cutaneous diseases. This laboratory studies how immune complexes are formed, how they cause tissue damage, and how they are cleared from the circulation. We have identified and partially characterized the immune complexes which exist in a variety of human diseases utilizing highly sensitive radioimmunoassays. We have developed a sensitive radioimmunoassay for the detection of IgA containing immune complexes. We have determined the antibody classes present in the immune complexes and examined the physiochemical characteristics of these complexes, as well as the reaction of these complexes with mediators of inflammation such as the complement system. We have performed the first prospective indepth immunological and immunopathological study of serum sickness in man. We have defined the time course of the clinical, immunological and immunopathpathological findings in human serum sickness. We have described a hitherto unknown cutaneous sign in humans specific for serum sickness. Since immune complexes may activate the complement system and since the complement fragments C5a and C3a are thought to be important in the pathogenesis of the inflammatory response in cutaneous and systemic diseases, we have purified C5a and studied its in vivo and in vitro reactivity. Its in vivo role was assessed by the first in-depth analysis of the cutaneous reactivity of this complement fragment in man. We have also studied the ability of C5a and C3a to modulate cell surface receptors for immunoglobulin and complement on the surface of leukocytes. Increasing evidence indicates that human endothelial cells, under certain circumstances, can be induced to become immunologically competent. In order to evaluate the role endothelial cells in immune complex mediated vasculitis we have isolated human umbilical vein endothelial cells, grown them in cell culture, examined them for the presence of immunologically relevant cell surface antigens and receptors before and after stimulation with soluble mediators of immunoregulation.

GPO 914-918

Other Professional Personnel:

Yasuo Kubota	Visiting Fellow	Derm NCI
J. Cason	Technician	Derm NCI

Project Description:

Objectives:

1) To further characterize circulating immune complexes in human diseases with regard to size, nature of the antigen, and subclass of antibody.

2) To develop reliable methods for specifically purifying immune complexes.

3) To continue clinical studies of immune complex diseases and reticuloendothelial system clearance function.

4) To evaluate in vivo the role of human C5a in the inflammatory response of normals and individuals with immunologically mediated diseases, and to conduct in vitro studies of C5a and C3a as modulators of the immune response. To directly compare the roles of C5a, C5a des Arg, C3a and C3a des Arg in inflammation.

5) To evaluate the role of human endothelial cells in immunologically mediated vascular damage.

6) To isolate and culture human dermal microvascular endothelial cells.

7) To evaluate the interactions between purified human anaphylatoxins and cultured human endothelial cells.

Material:

Serum, red blood cells, white blood cells and skin biopsies from patients and controls will be used. Human endothelial cells are isolated from umbilical veins and human foreskins. Purified human C1q, Raji cells, and ^{125}I (Bolton-Hunter reagent) and immunospecific antisera are used in the immune complex assays. Purified IgG fraction of anti Rh(D) human antiserum and ^{51}Cr. Human C5a and C3a are purified to homogeneity from human blood.

Methods Employed:

^{125}I-Clq binding assay, Raji cell IgG radioassay, Raji cell IgA radio-assay, direct and indirect immunofluorescence, Fc specific reticuloendothelial system clearance assay, column chromatography, sucrose density gradient ultracentrifugation, monoclonal antibodies, fluorescein activated cell sorter analysis, polyacrylamide gel electrophoresis, western blotting, cell culture.

Major Findings:

1) Patients with the blistering skin disease, dermatitis herpetiformis have IgA deposits in their skin which are exclusively IgA_1.

2) Dermatitis herpetiformis patients have IgA circulating immune complexes. These soluble immune complexes contain both IgA_1 and IgA_2.

3) Gluten is not detectable as an antigen in the circulating immune complexes of patients with dermatitis herpetiformis despite the fact that these patients have a gluten sensitive enteropathy.

4) Human C5a has been purified to homogeneity while retaining full biologic activity. In vitro studies have shown C5a to be a potent chemotactic agent, to degranulate polymorphonuclear neutrophils and aggregate platelets.

5) Human C5a is a potent mediator of cutaneous inflammation. It causes the rapid onset of a wheal and flare reaction when injected into normal skin.

6) Injections of human C5a into normal skin cause the accumulation of polymorphonuclear neutrophils around the blood vessels in the superficial and mid dermis.

7) The wheal and flare reaction associated with C5a skin tests can be decreased by pretreatment of the individual with antihistamines but not by pretreatment with systemic corticosteroids.

8) C5a causes the induction of FcIgG, C3b and iC3b receptors on human peripheral blood monocytes and polymorphonuclear neutrophils in a dose dependent fashion.

9) The immediate wheal and flare response to human C5a is mediated in part by circulating blood elements. In vivo studies in patients with aplastic anemia shown that these patients have decreased wheal and flare reactivity to C5a but not to histamine or morphine. The decreased responsiveness to C5a is proportional to the extent of leukopenia and thrombocytopenia.

10) Purified human keratin intermediate filaments (KIF) can be digested by proteolytic enzymes present in human skin.

11) The purified KIF which are insoluble in physiologic solutions are solubilized by digestion with trypsin, chymotrypsin, plasmin and urokinase.

12) The KIF digestion fragments are still reactive with human anti KIF autoantibodies.

13) The KIF digestion products are immunogenic in rabbits and the resultant antibodies react with both KIF digestion fragments and whole KIF.

14) Patients with aplastic anemia were treated with infusions of horse antithymocyte globulin and monitored, in a prospective fashion, for the clinical, immunological and immunopathological signs and symptoms of serum sickness. Almost all patients studied developed serum sickness. These included fever, malaise, cutaneous eruptions, arthralgias, G-I upset, proteinuria and lymphodenopathy.

15) Most patients who develop serum sickness also develop a unique cutaneous sign, thought to be specific for serum sickness. This cutaneous sign, which has not been previously described, consists of a band of erythema along the sides of the hands, feet, fingers and toes. It is often the earliest sign of serum sickness.

16) Patients who develop serum sickness also develop large amounts of circulating immune complexes. The levels of the immune complexes reach their peak at the time of peak clinical disease severity (days 10-14).

17) Patients who develop serum sickness have marked decrease in their serum complement levels coincident with clinical serum sickness and inversely related to levels of circulating immune complexes. These patients also have greatly increased levels of the circulating anaphylatoxin C3a during serum sickness.

18) Lesional skin from most patients with serum sickness shows deposits of IgM, IgA, IgE and C3 in the walls of small cutaneous blood vessels.

19) Human umbilical vein endothelial cells do not possess receptors for FcIgG or C3. Infection with herpes simplex virus will induce FcIgG, C3b and iC3b receptors but not C3d or FcIgM receptors on endothelial cells.

Bibliography:

Yancey, K.B., Hall, R.P., Lawley, T.J.: Pruritic urticarial papules and plaques of pregnancy: Clinical experience in 25 patients. J. Am. Acad. Dermatol. 10:473-480, 1984.

Yancey, K.B., and Lawley, T.J.: Circulating immune complexes: Their immunochemistry, biology and detection in selected dermatologic and systemic diseases. J. Am. Acad. Dermatol. 10: 711-731, 1984.

Hintner, H., and Lawley, T.J.: Anti stratum corneum antibodies are directed against keratin intermediate filaments. J. Invest. Dermatol. 82: 491-495, 1984.

Hall, R.P., Leiserson, W.M., Chused, T.M., Lawley, T.J.: Characterization of T-lymphocyte and monocyte populations in patients with dermatitis herpetiformis and HLA-B8/DRw3 normal individuals. J. Invest. Dermatol. 82: 231-234, 1984.

Lawley, T.J., Bielory, L., Gascon, P., Yancey, K.B., Young, N.S., Frank, M.M.: A prospective clinical and immunologic analysis of serum sickness in man. N. Engl. J. Med. 311: 1407-1413, 1984.

Hall, R.P., Gerber, L.H., Lawley, T.J.: IgA immune complexes in patients with psoriatic arthritis. Clin. Exp. Rheum. 2: 221-225, 1984.

Yancey, K.B., Hammer, C.H., Harvath, L., Renfer, L., Frank, M.M., Lawley, T.J.: In vivo studies of C5a as a mediator of human cutaneous inflammation. J. Clin. Invest. 75: 486-495, 1985.

Hintner, H., Neises, G.R., Lawley, T.J.: In vitro processing of extracellular human keratin intermediate filaments: enzymatic degradation and autoantibody interaction. J. Invest. Dermatol. 84: 108-113, 1985.

Straus, S.E., Tosato, G., Armstrong, G., Lawley, T.J., Davey, R., Preble, O., Brus, I., Blaese, R.M.: Persisting illness and fatigue in 23 adults: serologic and immunologic evidence for chronic active Epstein-Barr virus infection. Ann. Int. Med. 102: 7-16, 1985.

Lawley, T.J., Bielory, L., Gascon, P., Yancey, K.B., Young, N.S., Frank, M.M.: A study of human serum sickness. J. Invest. Dermatol. (In press).

Minuk, G.Y., Angus, M., Brickman, C.M., Lawley, T.J., Frank, M.M., Hoofnagle, J.H., Jones, E.A.: Abnormal clearance of immune complexes from the circulation of patients with primary sclerosing cholangitis. Gastroenterology (In press).

Romani, N., Hintner, H., Lawley, T.J.: Immunoelectronmicroscopic identification of upper cytoplasmic antigens on keratin intermediate filaments. J. Invest. Dermatol (In press).

Yancey, K.B., O'Shea, J., Chused, T., Brown, E., Takahashi, T., Frank, M.M., Lawley, T.J.: Human C5a modulates monocyte Fc and C3 receptor expression. J. Immunol. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03638-16 D

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of DNA Repair in Human Degenerative Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

J.H. Robbins Senior Investigator Derm NCI

COOPERATING UNITS *(if any)*

Biostatistics Branch, DCCP, NCI.

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.0	2.8	4.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors B

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies in this laboratory are designed to elucidate the role of DNA repair processes in human diseases and in carcinogenesis and in normal and abnormal aging. Most studies have been conducted with cells from patients with xeroderma pigmentosum (XP) who have defective DNA repair plus multiple cutaneous malignancies and premature aging of sun-exposed skin and of the nervous system. Cells from patients with diseases with abnormal cell growth and differentiation, and with primary neuronal, muscular, and retinal degenerations are also being studied. These diseases include ataxia telangiectasia, Alzheimer disease, Parkinson disease, Huntington disease, Duchenne muscular dystrophy, retinitis pigmentosa, and Cockayne syndrome. These studies are designed to elucidate the pathogenesis of these disorders. We assess the biological effectiveness of DNA repair primarily by in vitro assays of cell survival after treatment of the cells with the DNA damaging agents. We search for DNA damage by extracting the DNA and having it subjected to analysis by capillary gas chromatography-mass spectrometry.

GPO 914-918

Other Professional Personnel:

M.L. Dirksen	Expert	Derm NCI
A.N. Moshell	Medical Officer	AMSD, NIADDK
L.R. Seguin-Spillman	Staff Fellow	Derm NCI
R.E. Tarone	Mathematical Statistician	B NCI
K.H. Kraemer	Senior Investigator	CH, NCI
R. Polinsky	Senior Investigator	MN, NINCDS, NIH
L.E. Nee	Clinical Genetics Res. Assoc.	LCS, NINCDS, NIH
K.W. Liao	Visiting Fellow	Derm NCI
H. Jiang	Visiting Fellow	Derm NCI
J. Beer	Research Biophysicist	Ctr. Devices Rad. Health, FDA
D.S. Bergtold	Visiting Scientist	Ionizing Rad. Competency Program, NBS
M.M. Dizdaroglu	Visiting Scientist	Ionizing Rad. Competency Program, NBS
J. Nove	Research Specialist	Laboratory of Radiobiology, Harvard School of Public Health
R.J. Crouch	Senior Investigator	LMG, NICHHD

Project Description:

Objectives:

To study DNA repair processes in normal cells, cells from patients with each of the nine genetic forms of xeroderma pigmentosum (XP), and cells from patients with other diseases in which defective DNA repair is claimed or suspected, including degenerative diseases involving the somatic and/or autonomic nervous systems, skeletal muscle, and the retina; to determine the relationship between the function of such repair processes and 1) carcinogenesis, mutagenesis, cytotoxicity and 2) the clinical findings of photosensitivity, premature aging, and UV-carcinogenesis.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Materials:

Cells are obtained from established cell repositories and from hospitalized patients and outpatients at the NIH. Cells currently under study are dermal fibroblasts and Epstein-Barr virus-transformed lymphoblastoid cell lines.

Methods Employed:

Fibroblast and lymphoblastoid lines are cultured in the absence of antibiotics in laminar flow hoods.

The survival of fibroblasts after irradiation with UV or X rays, or after treatment with chemicals, is performed by counting the number of colonies which form. The survival of lymphocytes is determined by their ability to exclude the vital dye trypan blue. Unscheduled DNA synthesis is determined by determining autoradiographically the UV or X-ray-induced incorporation of tritiated thymidine. The chemical changes induced in DNA of cultured human cells are identified by extracting the DNA, separating the damaged basis by capillary gas chromatography, and identifying the products by mass spectrometry. Somatic cell genetic studies are being undertaken to determine complementation groups among the radiosensitive degenerative diseases. The ability of X rays to induce abnormal numbers of chromosome aberrations in the patients' cells is being studied by conventional cytogenetic techniques. DNA damage is being induced in the patients cells under conditions to maximize the difference in repair of the damage between patient and normal cells. These special conditions involve the use of new DNA-damaging chemicals and the use of confluent status of the cells being irradiated. The most useful DNA-damaging chemicals will be made radioactive. After their combination with the DNA of the cultured cells, the rates of removal of the various DNA lesions will be determined by appropriate physicochemical techniques (e.g., high performance liquid chromatography). Impaired removal of specific lesions would be evidence of defective repair of those lesions.

Major Findings:

1. We have determined the survival after exposure to UV radiation of a lymphoblastoid line from a 13-year-old boy with XP (XP1WI). With Dr. Ronald Polinsky we have evaluated this patient's neurological status and found him to have certain mild abnormalities consistent with those of XP. The survival of his irradiated lymphoblastoid cells in vitro correspond to those of other XP patients whose neurological abnormalities became clinically manifest after childhood. Our clinical and laboratory studies of this case support our DNA-damage hypothesis put forth to explain the basis for the premature death of neurons in XP.

2. In conjunction with Dr. J. Beer of the Center for Devices and Radiological Health, FDA, Rockville, MD, we have been studying the effect of chronic (i.e. low dose rate) X-irradiation of fibroblasts maintained at 37°C in a tissue culture incubation. We have found that there is no significant enhancement in survival of logarhymically growing normal fibroblasts when chronic irradiation (e.g. 1.0 rad/min) is used in place of standard acute dose rates (e.g. 126 rad/min.). However, with confluent fibroblasts an enhanced survival is obtained with the chronic dose rates. These results suggest that the state of confluence permits DNA repair processes to remove potentially lethal damage. Studies can now be conducted on fibroblasts from patients to determine if the patient's cells lack repair of potentially lethal damage.

3. We are attempting to test the survival of more lines from certain disease groups which we have previously shown to be radiosensitive. We have recently tested a fifth Down Syndrome line in our lymphoblastoid line trypan-blue X-ray survival test. It was equally as hypersensitive as the original four lines tested. Furthermore, we are applying our survival test to more ataxia telangiectasia heterozygote lines to see if they will all be distinguishable from normal. The three additional heterozygote lines tested bring to a total of eight the

number of heterozygote lines we can distinguish from normal. This study is being done in collaboration with Dr. K.H. Kraemer, Laboratory of Chemical Carcinogenesis, NCI, who is supplying the patient cell lines. Since it is believed that 1% of the general population may be ataxia telangiectasia heterozygotes and that they may comprise up to 5% of all persons dying from certain cancers before age 45, it would be extremely important to have a test which could identify such individuals.

4. We are collaborating with J. Nove, and J.B. Little, Laboratory of Radiobiology, Harvard School of Public Health, in studies to determine whether the radiosensitivity diseases we have detected in our lymphoblastoid line survival assay are radiosensitive also in fibroblast assays developed by Nove. The studies are conducted on cell lines we supply, and the data analysis is performed using methods we have developed. Nove has detected radiosensitivity to acute doses of X-rays, as well as to chronic doses of tritiated water, in our Usher syndrome lines.

5. Cytogenetic studies are being conducted to determine if DNA-damaging agents induce abnormal numbers of chromosome aberrations in human cells which have a hypersensitivity to the lethal effects of such agents. The development of such a cytogenetic test would make it possible to detect hypersensitivity to DNA-damaging agents within a few days, as opposed to the currently employed colony-forming test which requires two to three weeks. Cells from patients with ataxia telangiectasia, tuberous sclerosis, Alzheimer and other diseases are being studied. We have detected in ataxia telangiectasia heterozygote fibroblasts an abnormally high number of chromosomal abberations after G2-irradiation of the cells with X-rays.

6. In collaboration with Dr. Polinsky and Ms. Nee we are obtaining skin biopsies and blood samples from clinically well-characterized patients with premature death of nerve cells. Complete family pedigrees and neurological evaluations of the patients are also being obtained. A primary goal of this collaboration is to obtain several families with dominantly inherited Alzheimer disease so that we will be able to determine if this hereditary form of the disease has the radiosensitivity we have found in sporadic Alzheimer disease.

7. Ribonuclease H is an enzyme which hydrolyzes hybrid DNA (DNA:RNA). Hybrid DNA is apparently required for the initiation of DNA replication for semi-conservative DNA synthesis. It is not known whether or not hybrid DNA and RNase-H also play a role in repair replication of damaged DNA. In conjunction with Dr. R. Crouch, Laboratory of Molecular Genetics, NICHHD, we have demonstrated the presence of R-Nase-H in cultured human fibroblasts and lymphoblastoid lines. Studies are being conducted to determine if R-Nase-H is inducible by DNA-damaging agents, a positive finding of which would indicate a putative role of the enzyme in DNA repair.

8. We have analyzed the clinical history and autopsy slides of a Cockayne syndrome patient whose affected sibling's lymphoblastoid line has been studied by us for hypersensitivity to UV radiation. The patient's cell survival was the same as that of other Cockayne syndrome patients, even though this Cockayne syndrome kindred had one of the most severe forms of the disease ever reported.

found among patients with this DNA-repair-defect disease may be due to different amounts of incipient damage to DNA and not to differences in the capacity to repair DNA damage.

9. With Drs. D.S. Bergtold and M.M. Dizdaroglu, of Dr. Michael Simic's Ionizing Radiation Competency Program, National Bureau of Standards, Gaithersburg, MD, we are attempting to detect and identify specific forms of damage to DNA by means of capillary gas chromatography combined with mass spectrometry. Dr. Dizdaroglu can successfully identify with these techniques numerous types of damage in purified calf thymus DNA. In this joint venture between our laboratories, we are attempting to detect such damage (and its repair) in the DNA of human cells we treat with X-rays.

Significance to Biomedical Research and the Program of the Institute:

UV-radiation and ionizing radiation are causes of human cancer. Patients with XP are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor development in XP patients would, therefore, elucidate the role of DNA repair in preventing in normal humans those cancers which may be due to certain chemical and physical carcinogens. Extension of this work to include study of diseases in which DNA repair may be defective, such as those with hypersensitivity to ionizing radiation, will increase our understanding of the relationships between DNA repair processes and carcinogenesis.

XP patients' sun-exposed skin ages much more rapidly than normal humans' skin. Some XP patients develop neurological abnormalities that are due to the premature death of neurons. Our studies of the DNA repair defects are providing an understanding of the relationship of DNA repair processes to these aging phenomena in XP. Thus, our study of aging in XP organs is providing important knowledge as to how properly functioning DNA repair processes prevent such aging in normal humans. Our studies of other diseases with premature death of nerve, muscle, and retinal cells are also providing an understanding of such abnormal aging phenomena and of the relationship between the ionizingradiation-type of DNA damage and carcinogenesis.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Publications:

Robbins, J.H., Otsuka, F., Tarone, R.E., Polinsky, R.J., Brumback, R.A., and Nee, L.E.: Parkinson disease and Alzheimer disease: Hypersensitivity to X-rays in cultured cell lines. J. Neurol. Neurosurg. and Psychiatry. (In press).

Leech, R.W., Brumback, R.A., Miller, R.H., Otsuka, F., Tarone, R.E., and Robbins, J.H.: Cockayne Syndrome: Clinicopathologic and Tissue Culture Studies of Affected Siblings. J. Neuropathol. Exptl. Neurol. (In press).

Otsuka, F., Tarone, R.E., Seguin, L.R., and Robbins, J.H.: Hypersensitivity to ionizing radiation in cultured cells from down syndrome patients. J. Neurol. Sci. (In press).

Osuka, F., Robbins, J.H.: The cockayne syndrome: an inherited multisystem disorder with cutaneous photosensitivity and defective repair of DNA. Comparison with xeroderma pigmentosum. Am. J. Dermatopathology. (In press).

Robbins, J.H., Brumback, R.A., Polinsky, R.J., Wirtschafter, J.D., Tarone, R.E., Scudiero, D.A., Otsuka, F.: Hypersensitivity to DNA-damaging agents in abiotrophies: a new explanation for degeneration of neurons, photoreceptors, and muscle in Alzheimer, Parkinson and Huntington diseases, retinitis pigmentosa, and duchenne muscular dystrophy. In Molecular Basis of Aging, Basic Life Science Series, Edited by A.D. Woodhead, A.D. Blackett, V. Pond and A. Hollander, Plenum Press (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03667-01 D

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecules Defined by Autoantibody - Mediated Skin Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

John R. Stanley, M.D., Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.5	.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The general and long-term goal of my laboratory is to study autoantibody-mediated skin diseases in order to further our understanding not only of the pathophysiology of these diseases but also of the structure and function of normal epidermis and epidermal basement membrane zone (BMZ). Specifically, antibodies in these diseases define molecules in the normal epidermis. We have characterized the antigens defined by three of these diseases: bullous pemphigoid (BP), pemphigus vulgaris (PV), and pemphigus foliaceus (PF). We have defined the cells which synthesize these antigens, as well as the antigen defined by the autoantibodies found in patients with epidermolysis bullosa acquisita (EBA). We have demonstrated that BP antigen is distinct from other known BMZ molecules such as laminin and type IV collagen. Using these three molecules we have studied the BMZ of basal cell carcinoma and other epidermal tumors. We have also used these molecules to map, at a molecular level, the split in the BMZ in various hereditary mechanobullous diseases (epidermolysis bullosa). We have used the binding of antibodies to specific molecules to make diagnoses of EBA or BP in various complicated cases of these diseases. We have demonstrated that PV and PF autoantibodies bind distinct molecules. All PV autoantibodies bind the same molecule. We have used this fact to diagnosis certain patients with PV. Almost half of PF autoantibodies bind to a 155 kd glycoprotein which we have shown to be identical to desmoglein I, a desmosomal core glycoprotein.

PHS 6040 (Rev. 1/84)

GPO 914-918

Other Professional Personnel:

Leena Koulu	Visiting Fellow	Derm NCI
Nurit Rubinstein	Visiting Fellow	Derm NCI

Project Description:

Objectives:

1) To characterize the molecules defined by autoantibodies in sera of patients with various autoimmune blistering diseases such as: BP, PV, PF, EBA, herpes gestationis, and cicatricial pemphigoid.

2) To determine the cell source of these molecules.

3) To determine the physiologic function of these molecules.

4) To relate these molecules to the normal structure of the epidermal BMZ and the epidermis.

5) To understand the relationship of these molecules to the pathophysiology of these blistering diseases.

6) To understand the involvement of these molecules in the pathophysiology of other skin processes and diseases, such as reepithelialization of skin wounds, mechanobullous diseases, and epidermal neoplasms.

7) To apply the knowledge of the molecules involved in these blistering diseases to the diagnosis of these diseases.

Material:

Skin biopsies from patients with various blistering diseases are used. Normal epidermis is obtained from suction blisters or from dermatomed slices of skin from amputations or autopsy material. Normal human epidermal cells are cultured. Sera from patients (and from normals, as controls) are used. Monkey tissues and human tissue (from autopsy) are used to determine distribution of antigens. Polyclonal antibodies are raised in rabbits, and monoclonal antibodies are raised in mice.

Methods Employed:

Direct and indirect immunofluorescence are used for diagnosing diseases, characterizing autoantibodies and determining the histologic location of antigens. Immunoelectron microscopy is used to determine the ultrastructural location of antigens. Cell culture, with metabolic radiolabeling is used to study synthesis of antigen, as well as to characterize it.

Immunoblotting is used to characterize antigen. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis are used to characterize and compare antigens.

Major Findings:

1) Patients with PV all have antibodies against the same molecule, a glycoprotein of 210 kd, with disulfide-linked chains of 130 kd and 80 kd.

2) Antibodies from patients with PF bind to a molecule different from PV antigen.

3) About half of patients with PF have autoantibodies to desmoglein I, a desmosomal core glycoprotein.

4) EBA antigen is synthesized by human keratinocytes and fibroblasts.

5) Almost all patients with BP have autoantibodies directed against the same molecule, a 230 kd protein, which we have termed "BP antigen."

Significance to Cancer Research:

A basic understanding of the BMZ is important in ultimately understanding the interaction of tumor cells with BMZ. For example, we have shown that basal cell carcinoma displays a specific defect in one BMZ molecule, the BP antigen. Autoimmune blistering diseases can be used as models of immunologic self-recognition, which may be important in tumor cell recognition. Epidermal-specific antigens are modulated with differentiation, and the expression of these antigens is changed in transformed cells. The loss of some of these antigens may influence the behavior of epidermal neoplasms.

Publications:

Stanley.J.R., Koulu, L., and Thivolet, C.: Distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies. J. Clin. Invest. 74: 313-320, 1984.

Koulu, L., Kusumi, A., Steinberg, M.S., Klaus-Kovtun, V., and Stanley, J.R.: Human autotantibodies against a desmosomal core protein in pemphigus foliaceus. J. Exp. Med. 160: 1509-1518, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03656-12 D

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.M. Steinert, Visiting Scientist, Dermatology Branch, NCI

COOPERATING UNITS *(if any)*
Experimental Pathology Branch, DCCP, NCI; Laboratory of Molecular Biology, DCBD, NCI; Laboratory of Physical Biology, NIADDKD

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.75	5.25	1.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The biosynthesis, structure and function of the polypeptide chains which comprise the subunits of the keratin filaments of normal human, murine and bovine epidermis are being investigated. The subunits polymerize in vitro into native-type filaments. The details of filament ultrastructure are being investigated using image analysis procedures of filaments examined by transmission electron microscopic and scanning transmission electron microscopic techniques. Model structures generated from these methods will be computationally tested for compatibility with other physico-chemical data and amino acid sequence studies of individual filament subunits. cDNA cloned probes that encode human and mouse epidermal keratins have been isolated and are being used to determine the amino acid sequences of the proteins, and to study the structure and expression of keratin genes. The 10nm filaments of fibroblasts, muscle cells and neuronal tissues have been shown to be structurally similar to, but immunologically different from keratin filaments. A histidine-rich basic protein isolated from human epidermis and the slightly different protein of mouse epidermis specifically aggregate keratin filaments and other 10nm filaments in a manner suggestive of an interfilamentous matrix component. cDNA cloned probes will be isolated to study their structure, expression and amino acid sequence.

GPO 914-918

Other Professional Personnel:

P.E. Bowden, Ph.D.	Visiting Scientist	Derm. NCI
X-M. Zhou	Visiting Fellow	Derm. NCI
W.W. Idler	Chemist	Derm. NCI
S.H. Yuspa	Branch Chief	Lab. of Cellular Carcinogenesis and Tumor Promotion, DCE, NCI
D. R. Roop, Ph.D.	Senior Staff Fellow	Lab. of Chemical Carcinogenesis and Tumor Promotion, DCE, NCI
A.C. Steven, Ph.D.	Visiting Scientist	Lab. of Phys. Biol., NIADDK
R.D. Goldman, Ph.D.	Professor	Dept. Antmy & Cell Biol. Northwestern Univ.
D.A.D. Parry, Ph.D.	Professor	Dept. of Physics and Biophysics Massey Univ., Palmerston North, New Zealand
B.L. Trus, Ph.D.	Senior Investigator	Division of Computer Research and Technology
R.D.B. Fraser	Division Chief	C.S.I.R.O., Protein Chemistry, Melbourne, Australia

Project Description:

Objectives:

1) To characterize the ultrastucture of keratin filaments polymerized in vitro from the subunits of the filaments isolated form normal human, murine and bovine epidermis.

2) To characterize the polypeptide subunits of keratin filaments isolated from various benign and malignant human and mouse epidermal diseases.

3) To study the structure and expression of human and mouse epidermal keratin genes by use of cloned cDNA probes complementary to keratin mRNAs.

4) To investigate the nature of the highly-specific interaction between epidermal keratin filaments and filaggrin isolated from the epidermis.

5) To study the structure and expression of mouse and human filaggrin and their precursors by use of cloned cDNA probes complementary to filaggrin mRNAs.

6) To investigate the detailed secondary, tertiary and possible quaternary structure of keratins and filaggrins by use of computational analyses of the primary amino acid sequence data.

7) To investigate the chemical, immunological and structural similarities between epidermal keratin filaments and 10nm filaments isolated from a variety of cell types, such as BHK-21, CHO, HeLa, and PtK1 cells grown in culture, of muscle and neuronal tissues.

8) To study the chemistry and structure of several intermediate filament associated proteins isolated from mouse epidermal keratinocytes and cultured BHK-21 cells.

Major Findings and Methods Employed:

1. The polypeptide chains which comprise the subunits of the keratin filaments of normal human, bovine and murine epidermis have been isolated and individually characterized by standard protein chemical techniques. The unfractionated mixture of polypeptides or combinations of two of the purified polypeptides spontaneously polymerize in vitro in dilute salt solution into filaments that are uniformally 80 A wide and up to 40 μm long. The polypeptide composition of these filaments, and their structure, based on electron microscopy and X-ray diffraction, are the same as the in situ keratin filaments. Other X-ray and stoichiometric data suggested that the filaments contain regions of coiled-coil α-helix, perhaps arranged in a 2-chain manner which is the basic building block of keratin filaments. This structural unit is about 20 Å wide and about 500 Å long.

2. In collaboration with Dr. R.D. Goldman (Dept of Anatomy & Cell Biology, Northwestern University, Chicago, IL) it has been shown that the 10nm filaments of a variety of epithelial and mesenchymal cell-types grown in culture are morphologically very similar to epidermal keratin filaments. Most of these studies have been done with the 10nm filaments of BHK-21 and CHO cells since these are readily available in large quantities, but similar comparative work is also or underway with the filaments of HeLa cells, and the neurofilaments isolated from cattle and squid giant axons. The single proteins vimentin and desmin, the principal intermediate filament subunits of fibroblasts and muscle cells, respectively, are capable of filament assembly by themselves in vitro; that is, they form homopolymer filaments. All keratin filaments in contrast, contain at least two demonstrably different subunits; that is they are obligate copolymer filaments. Preliminary studies suggest that certain neurofilament subunits are capable of homopolymer and/or copolymer filament assembly in vitro. Since all of these filaments are basically very similar (although subtley different), it is perhaps not surprising that combinations from different sources also form native-type filaments in vitro. Hybrid filaments containing subunits from epidermis + fibroblasts, epidermis + smooth muscle, bovine epidermis + mouse epidermis, etc, have been formed. Such filaments are termed heterologous copolymers. These observations mean that cells can modulate their 10nm filament composition with regard to specific functions. In support of this idea, BHK-21 cells contain two types of filament subunits, that characteristic of fibroblasts and that of muscle cells, which apparently copolymerize in situ to form a filament of properties intermediate between the two. The presence of 10 nm filaments in cells is obviously extremely important and the structural studies described below should provide insights into their function in normal and transformed cells.

In addition, recent experiments have shown that many types of intermediate filament subunits are attended by other non-filamentous proteins which we call associated proteins (IFAPs). Some of these appear like lamins of

nuclear pore complexes. These proteins are immunologically and chemically related to keratins and concurrent morphological studies with Dr. Goldman suggest they may serve as anchoring points for the cytoplasmic filaments onto the nuclear surface. Other IFAPs are very large proteins, probably greater than 300 kilodaltons (kD), and may serve as cross-linking proteins for the filament network in cells. We plan to perform detailed biochemical, immunological and structural analyses on certain isolated IFAPs in an attempt to understand how they may function in cells.

3. In collaboration with Drs. D.R. Roop (LCCTP, DCE, NCI), cDNA species complementary to mouse and human epidermal keratin subunits have been produced and cloned in the E coli plasmid victor pBR322. Cloned probes which respectively encode mouse keratins of 67, 60, 59, 55 and 50 kD and a human keratin of 67 kD and 57 kD in size have been identified. These cDNA species are being subjected to DNA sequence analyses by use of the Maxam-Gilbert procedures. Assignment of amino acid sequences to the DNA sequences will then be performed by application of the known genetic code. We have now determined complete sequences for the mouse 60 and 59 kD and human 67kD keratins, and have substantial amounts of incomplete data for the mouse 67, 55 and 50 kD keratins, and human 57kD keratin.

These deduced amino acid sequences are being analyzed to determine the higher orders of structure that they may assume in keratin filaments. This work is being done in collaboration with Dr. B.L. Trus (Computer Systems Lab, DCRT), Dr. A.C. Steven, (Laboratory of Physical Biology, NIADDKD) and Dr. D.A.D. Parry (Dept. of Physics and Biophysics, Massey University, Palmerston North, New Zealand). All of the keratins contain a large central domain, rich in α-helix that is of conserved size of about 311-314 amino acids long, with non-α-helical end domains of that size varies widely with the size of the subunit. Thus keratins, and presumably all other intermediate filament subunits, differ from one another firstly in the size of their end-domains. Analysis of the data also shows that the amino acid sequences of these enddomains varies widely among the keratins. The 67 and 59 kD keratins have long very glycinerich end domains (up to 60% glycine), whereas in the 60 kD keratin, these domains are 40% glycine. The 50 and 55 kD keratins contain much less glycine and are in fact rich in serine residues instead. Further sequencing will be necessary to identify if there are any systematic patterns in the nature of the sequences of the end domains that may correlate with the expression of subunits in cells or putative functions of the filaments in which the subunits occur.

Analyses of the α-helical sequences show that they all have a common secondary structure in that they form four extended tracts of coiled-coil α-helix with another keratin, and that these tracts are separated by α-helical but non-coiled-coil linkers. These α-helical regions form 2-chain coiled-coils. Detailed comparisons of the sequences of these coiled-coil tracts show that there are distinctly different types of sequences. Acidic keratins (mouse 59, 55, 50kD, human 57kD) form Type I sequences. Neutral-basic keratins (mouse 67, 60, human 67kD) form Type II sequences. We have also shown that the other intermediate filament proteins, vimentin, desmin and glial fibrillary acidic protein, Type III sequences. The neurofilament proteins form Type IV sequences. In other experiments in collaboration with Drs. Parry and Steven, it has been shown that in keratins, the 2-chain coiled-coil molecule consists of one Type I

protein and one Type II protein, that is, in a heterodimer. In contrast, the Type III and Type IV proteins all form homodimers. In all cases, the two chains of the coiled-coil molecule are in exact-axial register.

By studying the distributions of ionic changes along the 2-chain coiled-coil molecules, there are only a limited number of ways in which a pair of 2-chain molecules and associate to form a higher structure within the filament. These occur when the 2 molecules are (1) in parallel and in register; (2) antiparallel and in register; (3) parallel but half-staggered; (4) antiparallel and half-staggered. Digestion of filaments containing keratin subunits whose sequences are known generates 4-chain coiled-coil helical fragments. We are in the process of characterizing the structure(s) of these fragments in order to determine which of the four above possibilities is most likely. Determination of the structure of the four-chain molecule will provide important clues on how the filaments are assembled.

4. In collaboration with Dr. Roop, Dr. Johnson, who was formerly with this laboratory, we have isolated and fully characterized two keratin genes: the mouse 59 kD (Type I) and human 67kD (Type II). We will now further characterize several features of these. Firstly, enhancer sequences and/or other sequences involved in the expression of these genes in epidermis will be characterized. Secondly, Dr. Paul Bowden will set up a cosmid library of mouse and human genomic DNA in an attempt to determine how the keratin genes are physically placed within the genome-whether the keratin genes are linked, their chromosomal locations, etc. Thirdly, preliminary data suggests that the two keratin genes and possibly those of other Types may have evolved from a common ancestor. Further detailed analyses of the genes and comparisons with other intermediate filament L genes are in progress to explore this hypothesis.

5. In collaboration with Dr. A.C. Steven, attempts are underway to understand the higher orders of filament structure. Good negatively-stained images of filaments can be subjected to optical diffraction analysis to obtain information of prominently repeating structural elements. Many filaments have to be computationally straightened in order to eliminate "noise" introduced by the curvilinear shape of the negatively-stained filaments. Diffraction images are then computationally averaged to identify the prominent repeats, which should then provide clues as to how the protofilament units are assembled into the filament. Basic structural information on filaments is also derived from scanning transmission electron microscopy (STEM) of filaments. This technique will be performed at the N.I.H. STEM facility located in the Department of Biology of the Brookhaven National Laboratory (Dr. J. Wall, Director) in Upton, New York. This technique provides information on the mass of the filaments and clues on the numbers of protofilamentous units in relation to length.

We have studied a variety of different types of intermediate filaments assembled from purified subunits of a wide range of molecular weights. The data show that the filaments are polymorphic; that is they consist of at least two distinct and different mass classes. Analyses show that within each mass class, the filaments contain the same numbers of subunits per unit length of filament, irrespective of their subunit content or subunit mass. The principal mass class

consists of 31-33 subunits/50 nm of filament length. Another minor mass class consist of 22-24 subunits/50 nm. These findings mean that within these two mass classes, intermediate filaments probably have the same structures. The significance of existence of two mass classes is unclear, but may have enormously important implications in terms of the structure and functions of the filaments in cells.

In addition, STEM technology provides information on the shape of intact filaments. Transverse density scans of the digitized STEM images show that filaments are in fact 15-17 nm wide, rather than the 10 nm normally seen in negatively-stained images of the filaments. This extra width is due to the presence of lower density mass at the periphery of the filament, which is presumably not normally visualized by negative staining. Interestingly, the size of this periphery varies in relation to the mass of the subunits of which the filament is constructed.

All of these data seem consistent with and elaborate upon the information available from amino acid sequences. The STEM data support the notion of a structural element of constant size that is common to all intermediate filament subunits, which is the central α-helical rod domain, that confers the common building block to all filaments. Filaments differ in size from one another in the size of their less density peripheries in relation to the size of the variable non- α-helical end domains of the subunits. Thus these sequences and STEM data predict that the variable end-domains occupy superficial positions on the filament where they may play an important role in determining filament function.

It is expected that the application of this new technology to the study of keratin and other related intermediate filaments will for the first time enable the construction of working models for the filaments. In collaboration with Drs. Fraser, Parry and Steven, optical diffraction and STEM analysis will be performed on (1) intact filaments from variety of keratin sources as well as on the intermediate filaments of several cell types; (2) protofilamentous forms of these filaments, obtained by dissociation in low salt or in high concentrations of sodium citrate buffer, pH 2.6; (3) particles of intermediate size obtained during various early stages of filament assembly in vitro; and (4) native filaments obtained from various types of cells where possible to provide a direct comparison of in vitro and in vivo filament structural forms.

In collaboration with Drs. Parry and Steven, we will undertake model building studies in an attempt to understand the structures assumed by the peripheral end domain sequences of the keration filaments. In some respects, the glycine- and serine-rich sequences are similar to those of chicken-leg scale keratins for which a model structure has already been proposed. Preliminary data suggests the glycine-serine-rich sequences form loops, stabilized by H-bonding between the serine-hydroxyls, and in which the glycines protrude. Such a structure would be very hydrophobic and this is consistent with both the properties of the isolated keratins, as well as the filaments in cells.

6. Comparisons of the polyacrylamide-gel electrophoretic profiles of the keratin subunits obtained from abnormal human epidermis such as Darier's disease and lamellar ichthyosis with those of normal epidermis show prominent differences in

numbers and mobilities of bands. Also, the abnormal polypeptides show limited facility for polymerization in vitro. Therefore, there may be differences in the chemical structures of the proteins. Attempts are underway to identify such differences by comparisons of two-dimensional gel electrophoretic maps of the CNBr and NBS peptides of the proteins obtained from normal and abnormal epidermis. Filaments assembled in vitro from psoriatic epidermis are also abnormal, and interestingly, form 'paracrystalline' structures consisting of several filaments associated side-by-side in an apparantly ordered manner. This may be due to the presence of an additional protein that perhaps functions like the basic protein or may be a feature characteristic of the filament proteins of psoriatic epidermis. Studies are underway to characterize this phenomnon further. Such studies may provide important clues on how the subunits are arranged in normal intact keratin and other types of intermediate filaments.

7. Normal mouse epidermal cells grown in monolayer culture can be made to synthesize the normal complement of keratin polypeptides. These proteins have been isolated and characterized by standard protein chemical techniques as done earlier with the bovine proteins. The reason why the mouse keratins are also being studied is that this cell culture system is currently being used for studies of in vitro carcinogenesis. Since the keratins are the principal synthetic products of the cells, they will be used as specific markers for the studies on carcinogenesis. To this end, in collaboration with Dr. Stuart Yuspa, a specific radioimmune assay has been developed to follow the changges occurring during carcinogenesis. Preliminary experiments have revealed marked alterations in the synthesis of the keratin proteins during treatment with carcinogens such as TPA and other growth effectors such as vitamin A.

8. We have shown that a histidine-rich protein isolated from rat epidermis specifically aggretates epidermal keratin filaments from several species in vitro to form a highly-ordered fiber. Electron microscopy of such fibers reveals a pattern of filaments 70-80 A in diameter embedded in a darker-staining background, or matrix. This structure is typical of the "keratin pattern" seen in the fully-differentiated stratum corneum of the epidermis. This suggests strongly that the basic protein is the matrix protein of epidermis. Our work constitutes the first real evidence for and demonstration of the role of a matrix protein in the epidermis. The interaction between the basic protein and filaments is highly specific since other fibrous proteins do not form the ordered structures. Therefore, there are structural features unique to keration filaments which recognize the basic protein. One practical limitation of such studies has been the difficulty in isolation of the basic protein. In an effort to resolve this, we have developed a very simple method for isolating large quantities of the similar protein from mouse epidermal stratum corneum which functions in the same way as the rat protein. Its chemical and functional properties will now be studied in detail. Studies on the interaction between the basic protein and defined fragments of filaments and filament subunits are underway to characterize the nature and specificity of the associations between these two components in the epidermis. Interestingly, in certain diseases of the epidermis involving abnormal keratinization, such as psoriasis, the amount of the basic protein is greatly diminished from normal. There may be a relationship between the absence of the basic protein and presence of abnormal keratin filaments which could provide important information on the disease itself. Since this basic protein also aggregates the

intermediate filaments from all sources so far examined we have chosen for it a new functionally-specific name, filaggrin. Presumably the filaggrin recognises structural features common to keratin and intermediate filaments. Studies are underway to determine the physiological significance of this result; for example it is not yet known whether filaggrin-like proteins are present in fibroblasts etc.

Attempts will also be made to study the biosynthesis of the filaggrin in intact epidermis and epidermal cells in culture. Preliminary studies suggest that filaggrin is initially synthesized on a very high molecular weight protein (300 kD) which is subsequently processed to give filaggrin (26 kD). This suggests that there are multiple copies of 26 kD filaggrin in the large precursor. Further studies to characterize this will be to prepare cDNA cloned probes from human and mouse epidermal mRNA, followed by DNA sequencing of the clones.

9. Intermediate filament subunits are phosphorylated in vivo by cyclic nucleotide dependent protein kinases. Presumably cells regulate the stucture and/or function of the filaments in this way. Attempts to characterize this process will initially involve estimation of the amount of phosphate bound to subunits, its location and possible function in terms of filament assembly in vitro. Filament subunits of mouse and bovine epidermis, CHO and BHK-21 cells, smooth muscle and various neuronal tissues will be examined. The serinephosphate content will be estimated by reaction of subunits with methylamine.

Significance to Cancer Research:

The epidermis offers a unique opportunity for the study of tumors not only because of the prevelance of tumors in this tissue but also because of its accessibility. One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. The keratin filaments and filaggrin are the most prominent intracellular components of all epidermal cells and therefore a study of their chemistry, structure and biosynthesis in both normal and abnormal epidermis will be of profound importance thesis in both normal and abnormal epidermis will be of profound importance in studying tumors in this tissue. The production of these protein in a well defined cell culture system will facilate studies of carcinogenesis in vivo and in vitro.

Proposed Course of Project:

The DNA cloning and related experiments will be done by Drs. Zhou and Bowden and in collaboration with Dr. Roop. The biochemical, biophysical and electron microscopic studies of the structure of normal epidermal keratin filaments will be done in this laboratory. The computational image analysis studies of filaments will be done in collaboration with Drs. Steven, Parry and Fraser. The cell culture studies will be done in the Laboratory of Cellular Carcinogenesis and Tumor Protection in collaboration with Dr. Yuspa. Collaborative efforts with Drs. B. Trus, R.D. Goldman and D.A.D. Parry will continue in the areas described above.

Publications:

Johnson, L.D., Idler, W.W., Zhou, X.-M., Roop, D.R., and Steinert, P.M.: Structure of a gene for the human epidermal keratin of 67000 Da. Proc. Natl. Acad. Sci., U.S.A. 82: 1896-1900, 1985.

Steinert, P.M., Parry, A.D., Idler, W.W., Johnson, L.D., Steven, A.C., and Roop, D.R.: Amino acid sequences of mouse and human epidermal Type II keratins of 67,000 molecular weight provide a systematic basis for the structural and functional diversity of the end domains of keratin intermediate filament subunits. J. Biol. Chem. in press, 1985.

Krieg, T.M., Schafer, M.P., Cheng, C.K., Filpula, D., Flaherty, P., Steinert, P.M., Roop, D.R.: Organization of a Type 1 keratin gene: evidence for evolution of intermediate filaments from a common ancestor. J. Biol. Chem. in press, 1985.

Parry, D.A.D., Steven, A.C., and Steinert, P.M.: The coiled-coil molecules of intermediate filaments consist of two parallel chains in exact axial register. Biochem. Biophys. Res. Commun. 127:1012-1018.

Steinert, P.M., Steven, A.C., and Roop, D.R.: The molecular biology of intermediate filaments. Cell, in press, 1985.

Steinert, P.M., and Parry, D.A.D.: Intermediate filaments: conformity and diversity of expression and structure. Ann Review Cell Biol. 1, in press, 1985.

Steinert, P.M.: Isolation and characterization of keratin intermediate filaments: in Biochemistry of the Cytoskeleton, ed. by I. Yahora and K. Maruyama, Japanese Biochemical Society, 1985, in press. (Japanese).

Roop, D.R., Cheng, C.K., Toftgand, R., Stanley, J.R., Steinert, P.M., and Yuspa, S.H.: The use of cDNA clones and monospecific antibodies as probes to monitor keratin gene expression. Ann N.Y. Acad. Sci., in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03659-11 D

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Therapy of Skin Cancer, Disorders of Keratinization, and Cystic Acne

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

G.L. Peck, Senior Investigator, Dermatology Branch, NCI

COOPERATING UNITS *(if any)*

1) Clinical Chemistry Service, NIH, Bethesda, Maryland 20205
2) Molecular Disease Branch, NHLBI, NIH, Bethesda, Maryland 20205
3) Cancer Prevention Studies Branch, DCPC, NCI, NIH, Bethesda, Maryland 20205

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.7	3.7	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

D

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Oral isotretinoin was effective in the prevention of skin cancer and in the treatment of a variety of disorders of keratinization (lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris), and cystic acne. Oral etretinate was more effective and less toxic than isotretinoin in the treatment of the disorders of keratinization. A high initial dose followed by a low maintenance dose of isotretinoin was comparably effective but less toxic than previously used continuous high-dosage schedules in the treatment of cystic acne. Psychological testing revealed decreased anxiety and depression in cystic acne patients after treatment with isotretinoin. Isotretinoin and etretinate treatment led to small but significant elevations in plasma lipids and changes in lipoproteins, which were responsive to dietary management. Absorption of etretinate is greater with milk, as a source of longchain fatty acids, than with water. Administration of etretinate with milk vs. water yielded different ratios of drug to metabolite in the serum. Etretinate is stored in fat and persists in the serum after discontinuation of therapy; trace amounts have been detected after more than 3 years. One chronic toxicity, "retinoid hyperostosis," characterized by anterior spinal ligament calcification and osteophyte formation of vertebrae, has been observed in 80% of patients treated with long-term, high-dose isotretinoin. Sixty percent of acne patients with moderate doses of isotretinoin for 9 months also developed vertebral osteophytes. Five patients with xeroderma pigmentosum were entered into a cancer chemoprevention study using isotretinoin. Of 5 patients entered into a phase I fenretinide-cancer chemoprevention study, 3 were discontinued due to toxicity (drug reaction, night blindness).

GPO 914-918

Other Professional Personnel:

G.L. Peck	Senior Investigator	Derm NCI
E.G. Gross	Senior Investigator	Cancer Prevention Studies, Br., DCPC, NCI
J.J. DiGiovanna	Senior Staff Fellow	Derm NCI
G. Gantt	Registered Nurse	Derm NCI
L. Zech	Senior Investigator	Molecular Dis. Br., NHLBI
K. Kraemer	Senior Investigator	Cell Genetics Br., NCI
S. Poliak	Medical Staff Fellow	Derm NCI

Project Description:

Objectives:

To evaluate safety and effectiveness of new oral and topical agents particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne.

Agents:

1) Isotretinoin, (13-cis retinoic acid) 10, 20 and 40 mg capsules
2) Etretinate, An ethyl ester of a trimethylmethoxphenyl derivative of retinoic acid (RO-10-9359) 10, 25 and 50 mg capsules.
3) Motretinide, an ethylamide of a tremethylmethoxyphenyl derivative of retinoic acid, (RO-11-1430) in 0.1% cream and 0.3% gel.
4) Fenretinide, (4-hydroxyphenylretinade) 100 mg. capsules.

Materials:

1) Keratinizing Dermatoses

   A Ichthyosis
      a) lamellar ichthyosis
      b) X-linked ichthyosis
      c) ichthyosis vulgaris
      d) epidermolytic hyperkeratosis
      e) non-bullous congenital ichthyosiform erythroderma
   B Darier's disease
   C Psoriasis
   D Keratoderma palmaris et plantaris
   E Pityriasis rubra pilaris
   F Nevus comedonicus
   G Erythrokeratodermia variabilis
   H Netherton's syndrome
   I Hailey-Hailey disease
   J Lichen planus
   K Pachyonychia congenita
   L Dyskeratosis congenita

2) Basal cell carcinoma

A Nevoid basal cell carcinoma syndrome
B Sunlight induced basal cell carcinomas
C Arsenical induced basal cell carcinomas
D X-ray induced carcinomas
E Xeroderma pigmentosum

3) Cystic and Conglobate acne and hidradenitis suppuritiva

Methods Employed:

1) Disorders of Keratinization
a) Oral isotretinoin was given for 6 month courses of therapy at 1 mg/kg/day. The dose was gradually increased (up to 8 mg/kg/day) to tolerance.

b) Oral etretinate was also given for 6 month courses of therapy beginning at 0.5 mg/kg/day. The dose was increased up to 1.5 mg/kg day or to tolerance.

c) Topical motretinide cream, 0.1%, was given in a double-blind manner versus placebo to 9 patients with disorders of keratinization. Topical motretinide gel, 0.3% was similarly tested in 3 patients. Therapy was given twice daily for 4 to 8 weeks without occlusion.

2) a) Basal Cell Carcinoma
Oral isotretinoin was given for 6 month courses of therapy at 1 mg/kg/day. The dose was gradually increased (up to 8 mg/kg/day) to tolerance.

b) Patients with xeroderma pigmentosum are being treated continuously with oral isotretinoin for 2 years.

c) Patients with multiple basal cell carcinomas were treated with fenretinide, 800 mg/day.

3) Cystic acne
a) Patients, 16 years and older, with at least 10 cystic lesions were treated with oral isotretinoin in an initial pilot study (14 patients) or in a double-blind, randomized study against placebo (33 patients) or in a third study testing a high initial and low maintenance dosage schedule (40 patients) or in a fourth study testing the need for the low maintenance dose (72 patients).
b) In the pilot study oral isotretinoin was given for 4 month courses of therapy beginning at 1 mg/kg/day. The dose was gradually increased to tolerance.
c) In the double-blind designed protocol, treatment was begun at a dosage of 0.5 mg/kg/day and was only increased if there was a significant worsening of the disease.

d) In the "high-low" study, 20 patients with predominantly facial acne, were given a high initial dose of 1.0 mg/kg/day for either 2 weeks or 4 weeks and then were given a low maintenance dose of 0.25 mg/kg/day for the remainder of a 16 week treatment period. Similarly, 20 patients with predominantly trunk acne received a high initial dose of 2.0 mg/kg/day for either 2 or 4 weeks followed by a low maintenance dose of 0.5 mg/kg/day for the remainder of the 16 week course of therapy.
e) In the fourth study 3 groups of 24 patients received either a) a high-low dosage schedule consisting of 2.0 mg/kg/day for 2 weeks and 0.5 mg/kg/day for 14 weeks, b) a high-dosage schedule alone in which patients received 2.0 mg/kg/day for 2 weeks followed by placebo for 14 weeks, or c) a continous low dosage schedule consisting of 0.5 mg/kg/day for 16 weeks.

Major Findings:

1. 14 patients with treatment-resistant cystic and conglobate acne was treated for 4 months with oral isotretinoin, a synthetic isomer of naturally occurring all-trans-retinoic acid. The average maximum dose received was 2.0 mg/kg/day. 13 patients experienced comlete clearing of their disease; the other had 89% improvement, as determined by the number of acne nodules and cysts present before and after therapy. Prolonged remissions, currently lasting an average of 8 years after discontinuation of therapy, have been observed in all 14 patients. One patient developed two lesions 12 months after discontinuation of therapy. Therapy was resumed in his case and he is once again free of disease. The mechanism of action of 13-cis-RA in the therapy of acne probably involves a direct inhibitory effect of the drug on the sebaceous gland, and from forehead skin surface lipid folm analyses, which indicated sebaceous gland inhibition by significantly lower levels of squalene and wax esters during therapy. Additionally, there was an average 84% decrease in mean forehead sebum production as compared to pretreatment values. There was a complete return to pre-treatment values after discontinuation of therapy in the sebum composition studies but only a partial return to normal sebum production.

2. A study comparing isotretinoin at initial dose of 0.5 mg/kg/day versus placebo in a double-blind format in the treatment of cystic and conglobate acne has been completed. 17 patients who initially received placebo worsened to the point where the double-blind code was broken and treatment with isotretinoin was begun. There was an overall 57% increase in the number of cystic lesions in this group. 16 of these 17 patients then received isotretinoin with a resultant 97% improvement. An additional 17 patients who had been randomly assigned to receive initial therapy with isotretinoin had an overall 96% reduction in number of nodules and cysts. There were an average of 46 nodules and cysts before treatment, and 2 afterwards. The average maximum dose of isotretinoin received by all 33 patients was 1.2 mg/kg/day, or 90 mg/day. 22 of the 33 patients have become completely free of lesions and only 3 patients have more than 3 lesions remaining. Patients who typically responded rapidly to a low dose were female, with facial lesions, and with an average of 27 cysts pretreatment. Slower responders requiring higher doses were typically male, with chest and back lesions, and with an average of 67 cysts pretreatment. 21 patients received one 4-month course of therapy; 12 received 2 courses after a 2-month treatment-free interval. 4 patients who had

cleared completely after one course of therapy with isotretinoin had mild relapses after 4 to 12 months post-discontinuation. Three of these 4 patients had received only 0.5 mg/kg/day during their initial course of therapy, and all cleared completely with their second course. All other patients continue to have prolonged remissions ranging from 79 to 86 months, average of 82. These results indicate that the beneficial therapeutic response is not a placebo effect, that 0.5 mg/kg/day is an effective dose particularly for facial lesions and is also productive of the common side effects. Higher doses are frequently required for control of chest and back lesions. Continued healing after discontinuation of therapy indicates that lower doses or alternate dosage schedules may also be effective.

3. In the third acne study analyzing the effectiveness of a "high-low" dosage schedule, the results were as follows. At the first post-treatment follow-up visit: 1.0 mg/kg/day for 2 weeks group there was an overall 70% reduction in cysts with an 85% reduction of facial cysts, in particular. In the 1.0 mg/kg/day for 4 weeks group there was an overall 82% improvement with an 89% response on the face. In the 2.0 mg/kg/day for 2 weeks group there was an overall 86% improvement with a 96% improvement on the face, 81% on chest, 83% on the back. In the 2.0 mg/kg/day for 4 weeks group there was an over all 77% improvement with 95% improvement on the face, 77% on the chest, and 63% on the back. Both 1.0 mg/kg/day groups had temporary, slight increases in numbers of observed cysts during the first 2 weeks of therapy, whereas the 2.0 mg/kg/day groups did not. We conclude that for most patients, especially for facial lesions, 2.0 mg/kg/day for 2 weeks is the optimal high dosage. In some patiens with back acne, the high initial dose of 2.0 mg/kg/day may have to be prolonged beyond 2 or even 4 weeks. The observed toxicity during the initial high dose periods was similar to our other studies. However, once the low maintenance dose was begun, there was a marked reduction initially in severity of toxicity and later in incidence. We conclude that except for a few patients with comparatively resistant back acne, the high-low dosage schedule is comparable in effectiveness to the continuous high dosage schedule of previous studies but is superior in minimizing the incidence and severity of observed toxicities.

4. In the fourth acne study, it was found that the high-low dosage schedule was superior to the initial high dose when used alone in the treatment of cystic acne. The high-low dosage schedule was also superior to the constant low dosage schedule in the treatment of cystic acne of the trunk. Cystic acne of the face responded well to both the high-low and the constant low dosage schedules. It was found that 13 of 22 patients receiving the highlow schedule, and 9 of 24 patients receiving only the high dose schedule, and 6 of 23 patients receiving the constant low dosage schedule had a 75% or greater reduction in total number of acne cysts at the end of the 16 week treatment period.

5. Prolonged partial suppression of quantitative sebum production was observed one year after discontinuation of a high initial, low maintenance dosage schedule for cystic acne. Twelve patients who responded partially to one 4 month course of isotretinoin were given a second course of therapy consisting of 2 mg/kg/day for 6 months. This group had a persistent 60% decrease in quantitative sebum production when measured one year or more after discontinuation of therapy. Patients who received and responded well to only one course of therapy had a

persistent 39% decrease from their original values when measured one year later. Dose-dependent, prolonged partial suppression of quantitative sebum production may be one mechanism by which prolonged remissions of cystic acne are induced by isotretinoin.

6. 12 patients with multiple basal cell carcinomal induced by sunlight, x-ray, arsenic, or the nevoid basal cell carcinoma syndrome were treated with oral isotretinoin. Of 270 tumors, 43 (16%) underwent complete clinical regression. Twenty-one of 35 of these tumors when biopsied after treatment were found to be gone microscopically as well. Correlation of therapeutic response withtumor size revealed that 19 of 83 (23%) tumors 3-5 mm in diameter and 18 of 99 (18%) tumors 6 to 10 mm in diameter underwent complete clinical regression, whereas only 6 of 88 (7%) tumors 11 mm or greater in diameter responded completely. Of the remaining tumors, 173 (64%) decreased in size and 54 (20%) were unchanged. Average maximum dosage in this group of 12 patients was 4.6 mg/kg/day with a range of 1.5 to 8.2 or 370 mg/day with a range of 120 to 660 mg/day. Duration of treatment varied from 16 to 96 weeks with an average of 56. Four patients had been sensitized and treated with DNCB six years previously. Three of these 4 patiens developed a marked inflammatory response in most tumors during therapy with oral isotretinoin. Two of the other 8 non-DNCB exposed patients developed final therapeutic response between these two subgroups with 21/109 (19%) tumors completely regressing in the DNCB treated group and 22/161 (14%) in the DNCB unexposed group. Most importantly, two patients in this group have received continuous courses of therapy for 7 to 8 years and have had no new tumors develop. The other patients who have either dropped out of the program or have had long intervals (greater than 8 months) between courses of therapy have had new lesions develop. We have concluded that isotretinoin may prevent or delay the development of tumors in this group of patients. Histologically, a dense small cell infiltrate was seen in tumors undergoing inflammation, suggesting that 13-cis-RA could either be enhancing a host immunologic response or be producing a direct cytotoxic effect on the tumor.

7. 57 patients with cutaneous disorders of keratinization were treated with oral isotretinoin. Diseases included Darier's disease (DD) (9 patients), lamellar ichthyosis (LI) (10), psoriasis (PSOR) (9), pityriasis rubra pilaris (PRP) (5), keratoderma palmaris et plantaris (KPP) (4), epidermolytic hyperkeratosis (EHK) (4), non-bullous congenital ichthyosiform erythroderma (NBCIE) (3), x-linked ichthyosis (XLI) (3), Hailey-Hailey disease (HH) 2), and 1 each with a variant form of NBCIE, erythrokeratodermia variabilis (EKV), pachyonychia congenita (PC), ichthyosis vulgaris (IV), netherton's syndrome (NS), and porokeratosis (PORO). The patients ranged in age from 4 to 82 years. The dosage varied from 0.5 to 8.2 mg/kg/day, the duration of treatment varied from one week to over 9 years. The average maximum dosage was 160 mg/kg/day or 2 mg/kg/day. Treatment was initially given in 16 week courses of therapy with intervening 8 week treatment-free intervals. Now it is given in 6 month courses with 1-4 week intervals. Good or excellent responses were seen in DD (7), LI (8), NBCIE (3), PSOR (4), KPP (2), EKV (1), and IV (1). Partial responses were observed in EHK (3), PRP (2) , LI (1), DD (2), KPP (10, PC (1, variant-NBCIE (1). Patients showing minimal or no response included: PSOR (5), XLI (3), LP (2), HH (2), NS (1), EHK (1), PRP (1), KPP (1). One patient with PORO and one with LI stopped treatment within one week before a therapeutic evaluation could be made. The mechanism by which this

synthetic retinoid alters these disease states is not known but may be related to the observed ability of vitamin A to affect epithelial differentiation. The observed variation in therapeutic response could be related to the presence or absence of specific retinoid binding proteins. Our results indicate that synthetic retinoids, such as isotretinoin may represent a potent new class of drugs in the treatment of cutaneous disorders of keratinization, several of which were previously treatment-resistant.

8. 75 patients with cutaneous disorders or keratinization were treated with etretinate, an oral synthetic aromatic retinoid which is an ethyl ester of a trimethyl-methoxyphenyl derivative of retinoic acid. Diseases included DD (18), PSOR (23), PRP (10), LI (6), EHK (4), IV (3), KPP (2), variant-NBCIE, NBCIE (1), Kyrle's dis(1), PC (1), HH (1), PORO (1), and LP (1). The average maximum dosage was 86 mg/day or 1.2 mg/kg/day. Duration of treatment varied from 1 week to over 6 years. Treatment was initially given in 16 week courses of therapy with 8 week treat-free intervals. Now it is given in 6 month courses with 1-4 week intervals. Good or excellent responses were seen in PSOR (8), DD (7), PRP (4), Kyrle's (1). Minimal responses were noted in one patient with PROP, the lingual lesions of PC lesions of PC (1), and the oral lesions of LP (1). Worsening was observed in HH (1). Response to therapy was initially variable in psoriasis. 12 patients worsened for 8, 12, or even 16 weeks of therapy and then improved; 11 other patients with psoriasis improved immediately. All other patients with responsive diseases improved immediately upon beginning therapy. Etretinate is clearly superior to isotretinoin in the treatment of psoriasis, IV, KPP, EHK.

9. 9 patients with disorders of keratinization were treated with topical motretinide crea, 0.1% in a double-blind manner against placebo. Diseases included PSOR (3), KPP (2), EHK (1), and variant NBCIE (1). No patient exhibited a preferential beneficial effect of the retinoid cream. 7 patients who received the retinid cream showed improvement indicating either a beneficial effect of the vehicle or an overall seasonal improvment in the patients disease. No systematic or local toxicities were observed. These results indicate that either motretinide is ineffective or that the 0.1% concentration is inadequate. Similar testing with the 0.3% gel also revealed no therapeutic or toxic effects.

10. Common side effects in most patients treated with these retinoids were limited to the skin and mucous membranes and included cheilitis, facial dermatitis, conjunctivitis, xerosis, dryness of the nasal mucosa with mild nosebleeds, and easy peeling of the stratum corneum upon trauma termed "skin fragility." Aside from skin fragility, the above side effects were either more common or more severe during treatment with isotretinoin than with etretinate. However, there were side effects that were present in higher incidence in patients treated with etretinate. These include: skin fragility, hair thinning (telogen), itching, palmar peeling, dry mouth with thirst, arthralgias, "stickiness" of the skin, and paraonychia. Laboratory abnormalities during therapy are limited to elevations of the erythrocyte sedimentation rate, temproary low-grade elevations of the transaminases in approximately 10% of patients which return to normal values without discontinuation of therapy, and elevations of the alkaline phosphatase, LDH, and serum triglycerides in a few patients.

In the "High-Low" cystic acne study described in Methods employed, section 3d, and in Major Findings, section 3 analyses of serum lipids were performed. In those 20 patients initially receiving 1.0 mg/kg/day of isotretinoin an increase of 20% in total plasma triglycerides and an increase of 6% in total plasma cholesterol was noted during therapy when compared to pretreatment values. In 9 of these 20 patients HDL-cholesterol was measured and found to be decreased by 12% during therapy. In 19 of the 20 patients initially receiving 2.0 mg/kg/day, the observed changes in serum lipids during therapy as compared to pretreatment values, were: 1) an increase of 24 mg/dl in HDL-cholesterol, 2) an increase of 5.4 mg/ dl in VLDL-cholesterol, 3) a decrease of 3.6 mg/dl of HDL-cholesterol, 5) 5) an increase of 25.5 mg/dl of total plasma cholesterol, and 5) an increase of 44.2 mg/dl of total plasma triglycerides. All values wre significant at levels of $p < 0.01$.

A third group of 9 patients were retreated with 2.0 mg/kg/day for 6 months. Data from each of these three different treatment schedules indicated no significant change in triglyceride or cholesterol values after the initial rise from base line values noted after the first week of treatment. Therefore, 2 mg/kg/day of isotretinoin over 6 months did not lead to significantly higher triglyceride or cholesterol levels than those observed with shorter treatment periods. All values returned to base line within 4 weeks of stopping patients with psoriasis treated with oral etretinate at a maximum dosage of therapy.

Similar changes in serum lipids and lipoproteins have been observed in 9 patients with psoriasis treated with oral etretinate at a maximum dosage of 1.0 mg/kg/day. These changes returned towards normal with dietary management and with decreased dosage.

In general, these side effects are dose-dependent in incidence and severity, relieved by adjunctive bland therapies, well-tolerated by the patients, and totally reversible upon discontinuation of therapy.

11. Fifty patients with various skin disorders have been treated with the synthetic retinoids, isotretinoin and etretinate. Back and neck stiffness was a frequent symptom that we investigated with vertebral spine films. Seventy-two sex-matched controls were selected for comparison with all treated, regardless of skin diagnosis. The differences in frequencies between these two groups of the defined abnormality used in this study (anterior spinal ligament calcification and isteophyte at 2 or more vertebral levels without disc space narrowing) were not significant. However, when patients with basal cell nevus syndrome or basal cell carcinoma who had never received retinoid were compared with those who had received isotretinoin at a high dose for a minimum of 2 years the differences were significant ($p < 0.01$). This study suggests a correlation between long-term ingestion of high-dose (minimum = 1.5 mg/kg/day) isotretinoin and development of an ossifying diathesis, termed "retinoid hyperostosis." This is the first chronic toxicity observed with oral retinoid therapy.

We subsequently reviewed 2 groups of our patients. One group consisted of 40 patients with cystic acne treated with isotretinoin at a mean dose of 1.3 mg/kg/day ($\pm$ 0.7) for a mean duration of 9 $\pm$ 3 months. The second group consisted of 11 patients with either multiple basal cell carcinomas or a disorder of

keratinization who were treated with isotretinoin at a mean dose of 1.6 mg/kg/day (± 0.7) for a mean duration of 52 ± 8 months. Spine X-rays were taken at least 6 months after discontinuing therapy in the acne group and at varying intervals of at least one year in the second group of long-term therapy patients. Evidence of osteophytic spurring of the vertebral spine was found in 60% of the acne group and in 82% of the second group. The mean number of osteophytes was 3 ± 5 in the acne group and 15 ± 10 in the second group. In both groups, older patients had more osteophytes and the areas of greatest involvement were lower cervical (C4-cervical range of motion which was probably related to osteoarthritic changes unrelated to isotretinoin therapy and not to the osteophytes. There was no change in vertebral spine function over one year in the second group despite small increases in number of osteophytes in patients continuing therapy. The prevalence of observed osteophytes appears to be related to the duration of therapy with isotretinoin.

12. To examine the association of synthetic retinoids within lipoprotein fractions, sera from 6 patients (psoriasis, Darier's disease, pityriasis rubra pilaris) receiving etretinate and from 6 receiving isotretinoin (basal cell carcinoma, acne) were separated into lipoprotein fractions by either ultracentrifugation or by heparin-manganese precipitation. Retinoid concentrations were measured with high pressure liquid chromatography. Sera were evaluated after a 12 hour fast and four hours after the patients ingested retinoid with whole milk. 80 ± 1.7% of the serum etretinate but only 27 ± 1.5% of the serum isotretinoin was found with the beta-lipoproteins. The lipoprotein bound etretinate was found in both the very low density lipoprotein and the low density lipoprotein fractions. When the fasting and four hour specimens wre compared, there were no differences in distribution of these retinoids within the betalipoprotein fraction or the albumin containing fraction, suggesting that these associations are independent of serum concentrations of the drug. No differences were observed between individuals despite differing ages and diseases. It is not known whether the beta-lipoproteins function to transport the retinoids to cell surface receptor sites or serve only as a circulating reservoir.

13. Since etretinate is lipid soluble and may be poorly absorbed in the absence of a fat load, we sought to determine whether diet affected its patients received a 1 mg/kg AM dose of etretinate with water or 1 pint of whole milk. Light protected serum samples were drawn at various times and analyzed for etretinate and its major metabolite (RO 10-1670) by high pressure liquid chromatography. Since low levels of etretinate and Ro 10-1670 persist after chronic administration, serum levels were corrected for this baseline (zero time) value in each set of assays. At every time period, the mean corrected serum etretinate concentration after administration with milk was higher than after administration with water. The mean corrected peak serum concentration of etretinate (2 to 6 hours) was significantly higher after administration with milk (98 ± 13 µg/dl) than water (29 ± 5 µd/dl). In each patient the peak serum etretinate after administration with milk ranged from 147% to 375% higher than after water. Over a 24 hours period there was an overall 296 ± 26% ($p<0.0005$) increase in serum etretinate after administration with milk compared to water in 5 patients with Darier's disease. In contrast, serum levels of the metabolite (Ro-10-1670) were found to be similar regardless of the mode of administration of etretinate. No difference in the pattern of absorption was observed between patients with Darier's disease

and psoriasis. These data indicate that the serum level of etretinate, but not its major metabolite can be markedly increased by administration of the drug with a fat load.

14. Retinol absorption occurs with long chain fatty acids (LCFA), mainly in association with lymph chylomicrons. Retinoic acid, in contrast, is absorbed into the portal system. An aromatic retinoid, etretinate, is present in higher concentrations in serum after administration with milk compared to water. We sought to determine if this is due simply to enhanced solubility or on etretinate therapy were given a 1 mg/kg morning oral dose of etretinate with either 1 pint of whole milk, water, 20 gms of medium chain triglycerides (MCT), or 1 oz. of ethanol. Milk without etretinate was also performed. Light-protected serum was drawn into evacuated tubes without anti-coagulant at 0 (baseline) and 4 hours-the usual peak serum concentration (PSC) time. All PSC's were corrected by subtraction of baseline. The mean PSC increased after administration of etretinate with either water, MCT or ethanol compared to milk alone ($p<0.003$) increases over the PSC's obtained after administration with water ($\Delta 99.5 \pm 18.3 \mu g/dl$), MCT ($\Delta 108.1 \pm 21.0 \mu g/dl$) or ethanol ($\Delta 107.1 \pm 19.5 \mu g/dl$). Mean PSC's after administration of etretinate with water, MCT or ethanol were not significantly different. 24 hour time course curves in one patient demonstrated that observed differences were not due to shifting of the peak time. This study indicates that enhanced etretinate absorption with milk is not solely due to the lipophilic milieu but requires LCFA and suggests that etretinate is predominately absorbed via the lymph rather thanthe portal route.

15. Prolonged blood levels have been observed as long as 3 years post-treatment, indicating significant body storage of this aromatic retinoid. Thus, it is not known with certainty when it is safe for a woman to conceive a child after she has been treated with etretinate. In this regard, one patient with Darier's disease did become pregnant during her first month of etretinate therapy. Etretinate was discontinued and the patient was informed of the potential risk to the fetus. She elected to continue the pregnancy and gave birth to a normal male infant.

16. A study testing the effectiveness of oral isotretinoin, 2.0 mg/kg/day for 2 years, in the prevention of skin cancer in xeroderma pigmentosum has been initiated recently. Six patients have entered this trial, beginning 11 months ago. The first patient was removed from the study to facilate treatment of a recurrent squamous cell carcinoma of the skin which was found to be invading the maxillary sinus. The remaining 5 patients are still enrolled in the study and are tolerating isotretinoin, 2 mg/kg/day. It is too early to determine if a definite chemopreventive effect has occurred. In addition to mild to moderate mucocutaneous toxicities, 2 instances of mild arthralgias, 4 instances of low-level triglyceride elevations, and 3 instances of low-level SGOT or SGPT elevations have occurred.

17. A phase I study testing fenretinide in the chemoprevention of basal cell carcinoma has begun. Five patients with multiple basal cell carcinomas were treated with fenretinide, 800 mg/day, for 9 days to 8 weeks. Three of the patients were removed from the study due to toxicity. One had a drug eruption with an multiforme-like erythema rash, diarrhea and low-grade elevations of liver

function tests. This reaction disappeared promptly within one week after the drug was stopped. Two patients had night blindness with abnormal dark adaptation tests and abnormal electroretinograms. Because of these toxicities, the remaining two patients were also removed from the study.

18. Psychological testing profile of mood scale, Hopkins Symptom Checklist, Rosenberg Self-Esteem Questionnaire, and the NIH Mood Scale) was performed in 71 cystic acne patients before and after treatment with isotretinoin. Patients showed significantly reduced anxiety and depression following treatment. Clinical interviews after treatment revealed lessened preoccupation with details of hygiene and concern about appearance and improvement in feelings of embarrassment and self-consciousness.

19. Of 72 patients with cystic acne who were participating in isotretinoin treatment protocols and had a history of minocycline therapy, 4 were found to have apparently permanent tooth discoloration. No other cause was found for this tooth discoloration, which in all likelihood is due to minocycline. This was unusual in 2 respects: (1) although tetracycline is known to stain teeth of children younger than 8 years of age, these pateints were in mid-to-late-adolescence, and (2) the staining appeared to be idiosyncratic and not dose-dependent since one patient developed discoloration after only one month of minocycline therapy.

Significance to Cancer Research:

a) These are exploratory studies of a new class of drugs (synthetic retinoids) in the treatment of skin cancer. Skin cancer should prove to be a valuable lesion for the screening of these agents because of tumors are observable, measurable, and therefore can be used as an objective indicator of response to therapy. Furthermore, the patients can be expected to be in good health, which would allow for long term studies.

b) The profound beneficial effect of isotretinoin in the treatment of acne and both retinoids in the treatment of cutaneous keratinizing diseases indicates that other keratinizing disorders of man, for instance, preneoplastic squamous metaplasia of tracheobronchial and urinary bladder epithelial, could be succes sfully treated with the synthetic retinoids. Treatment of these keratinizing dermatoses and acne may also provide useful information in the evaluation of newer and potentially more potent and less toxic synthetic retinoids.

Proposed Course of Project:

1) Continued treatment of patients with acne, basal cell carcinomas, and disorders of keratinization with isotretinoin including the establishment of a collaborative group to study the chemoprevention of basal cell carcinoma with isotretinoin. This is being done in collaboration with the Cancer Prevention Branch, DCPC, NCI.

2) Continued treatment of disorders of keratinization with oral etretinate.

3) Treatment of patients with xeroderma pigmentosum using oral isotretinoin to prevent skin cancer formation.

4) Testing of fenretinide in patients with skin cancer, acne psoriasis, and ichthyosis in collaboration with the Cancer Prevention Branch, DCPC, NCI.

Publications:

Peck, G.L.: Synthetic retinoids in dermatology, in The Retinoids, M.B. Sporn et al (eds), Academic Press, New York, 1984, pp. 391-411.

DiGiovanna, J.J., Peck, G.L.: Pityriasis rubra pilaris, in Current Therapy in Dermatology, T.T. Provost and E.R. Farmer (eds), B.C. Decker, Inc., Burlington, Ontario, Canada, 1985, pp. 8-13.

DiGiovanna, J.J., Gross, E.G., McClean, S.W., Ruddel, M.E., Gantt, G., and Peck, G.L.: Etretinate: Effect of milk intake on absorption. J. Invest. Dermatol. 82: 636-640, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03630-15 D

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of Vitamin A and Analogs on Chick, Mouse and Human Skin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

G.L. Peck, Senior Investigator, Dermatology Branch, NCI

COOPERATING UNITS *(if any)*

Dept. Dermatology, UCSF
Lab of Vision Research, NEI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors B

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project proposed to morphologically and biochemically define the mechanism of action of vitamin A and its derivatives (retinoids) in altering epidermal differentiation in normal skin, and in benign andmalignant lesions of skin. Topical all-trans retinoic acid, but not systemic 13-cis-retinoic acid, increased gap junction density and decreased desmosome density in treated basal cell carcinomas. This indicates that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms.

A specific cytosol retinol binding protein (CRBP) has been identified in mouse, normal human skin and skin and human skin from patients with Darier's disease, psoriasis and basal cell carcinomas. A specific cytosol retinoic acid binding protein (CRABP) has also been identified in newborn mouse and normal human adult skin and newborn foreskin. The qualitative and quantitative distribution between the epidermis and dermis of both CRBP and CRABP has been determined in adult human lower limbskin.

GPO 914-918

Other Professional Personnel:

J.J. DiGiovanna	Senior Staff Fellow	Derm, NCI
E.G. Gross	Senior Investigator	Cancer Prevention Studies Br., DCPC, NCI
P.M. Elias	Professor, Dept. of Dermatology	Univ. of California Medical Center
G. Chader	Chief, Lab of Vision Research	NEI

Project Description:

Objectives:

1) To investigate the mechanisms governing epidermal cell differentiation.

2) To morphologically investigate the mechanisms by which vitamin A and its derivatives alter epidermal cell differentiation in normal adult skin and in benign and malignant lesions.

The Objectives and Approach of the National Cancer Plan that this project most closely supports is 3.1: Study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Material:

1) 0.2-0.4 mm thick, Castroviejo keratotome slices of normal and diseased human skin. Skin from patients with Ichthyosis and Darier's Disease (NCI-3643) has been used to date.

2) 3-4 mm punch biopsies of normal and diseased human skin both treated and untreated with synthetic retinoids (NCI-3643).

Major Findings:

1) Freeze-fracture replicas and thin sections of cell membranes of: 1) 11 basal cell cancers (BCC) treated twice daily for two weeks with topical 1.0% all-trans retinoic acid (RA); 2) 21 BCC treated for 2 to 17 weeks with oral 13-cis retinoic acid (CRA) (1.0-8.0 mg/kg/day); and 3) 17 BCC prior to retinoid treatment and/or after applications of vehicle alone. Both thin sections and replicas were examined and photographed in a single-blind fashion, and the density and size distribution of gap junctions and desmosomes were computed planimetrically. Neither RA nor CRA treatment appeared to influence hemidesmosome or microfilament populations. Structural changes in both treatment groups did not correlate with either tumor regression or inflammation. This indicates that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms.

2) Sucrose density centrifugation was used to identify specific cytosol retinol (CRBP) and retinoic acid (CRABP) binding proteins in newborn mouse

skin. A similar amount of retinol binding in skin was found after a 15 minute, 37°C incubation compared to the standard 2 hour, 4°C incubation. Stability of binding during frozen storage was demonstrated for periods up to 4 1/2 months. The ability to augment retinol binding by 75°C with the addition of a 24-hour pretreatment of the tissue with lyophilization allowed the detection of binding in smaller quantities of tissue.

Epidermal-dermal separation of newborn mouse skin with trypsin and the amount of binding with 2 layers was determined. Equivalent amounts of retinoic acid binding and seven times more retinol binding were found in the dermis compared to epidermis.

Specific retinol and retinoic acid binding was also identified in adult human skin. Adult human skin separated with trypsin or EDTA revealed that the epidermis bound significant levels of retinol and retinoic acid while the dermis did not bind detectable levels. Adult human epidermis bound 10 to 20 times more retinoic acid than retinol. The ratio of Retinol/Retinoic Acid bound in adult human epidermis was similar to newborn mouse skin.

We now have identified CRBP in 0.3 mm keratomed normal adult human skin and examined the relative contribution of the epidermis and dermis to the total retinoid binding. CRBP and CRABP were measured as the binding of ^{3}H-retinol (^{3}H-ol) and ^{3}H-retinoic acid (^{3}H-RA) to cytosol preparations using sucrose gradient centrifugation. Saturability and specificity were determined by demonstrating the presence or absence of competition with an excess of nonradiolabeled retinoid. The mean specific activity of ^{3}H-ol and ^{3}H-RA binding (0.57 $\pm$ 0.07, 3.43 $\pm$ 0.57 pmol/mg protein) to cytosol preparations from different specimens of adult human skin was determined. Skin obtained from one sample was assayed for cytosol ^{3}H-ol and ^{3}H-RA binding as full thickness skin (0.36 $\pm$ 0.09, 2.11 $\pm$ 0.38 pmol/mg protein), EDTA-separated epidermis (0.36 $\pm$ 03, 3.69 $\pm$ 0.13 pmol/mg protein) and subjacent dermis (neither detectable). The epidermis alone demonstrated binding for each retinoid at least as great as the full thickness skin. Adult human skin was keratomed at 0.1, 0.2 or 0.3 mm, and histology was obtained. Increasing the thickness from 0.1 to 0.2 mm (which added almost all of the lower epidermis) increased the specific activity for both retinoids. As thickness increased to 0.3 mm (adding significant dermal contamination), the specific activities decreased. These results suggest that CRBP and CRABP in adult human skin are predominantly located in the epidermis.

Specific Retinol and Retinoic acid binding was identified by the skin of a patient with Darier's disease. Specific retinol binding was found in basal cell carcinoma and psoriasis.

3) Protein tyrosine kinase and protein phosphotyrosine phosphatase activities were measured in extracts of skin samples from patients with psoriasis. Both enzymatic activities were significantly greater in samples taken from involved area, characterized by epidermal hyperproliferation, than from adjacent skin of normal appearance. These data provide evidence that regulation of protein tyrosine phosphorylation and dephosphorylation is associated mitotic activity *in vivo*.

4) Etretinate, an aromatic retinoid under investigation for the therapy of psoriasis, may function by alteration of the rate of epidermal cell proliferation. Serial biopsies for mitotic index and sera for retinoid concentrations were obtained from patients at the onset of a course of etretinate therapy to assess for alteration in epidermal proliferation.

Significance to Cancer Research:

1) Since carcinogenesis is an instance of altered differentiation, studies of vitamin A effects on differentiation, may serve as an excellent model for investigations of cellular control mechanisms which relate to carcinogenesis. The fact that carcinogenesis is influenced very markedly by vitamin A deficiency directly links research on the epithelial effects of vitamin A to cancer research.

2) Furthermore, retinoids are of value in the treatment of malignancy. The mechanisms of action of retinoids in affecting differentiation may be related to its antineoplastic activity.

Proposed Course of Project:

1) Continued ultrastructural examination of normal skin and benign and malignant lesions of skin both treated and untreated with synthetic retinoids.

2) Continued study of the specific mechanism of vitamin A and retinoid binding to normal and diseased skin with emphasis on elucidating the mechanism of action of these drugs on skin. The distribution and density of specific receptors for retinol and retinoic acid in normal and diseased skin will be studied.

Publications:

None.

ANNUAL REPORT OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

The clinical research program of the Metabolism Branch is directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. In this area, a broad range of immunological investigations are carried out in patients with immunodeficiency diseases and a high incidence of neoplasia, as well as in patients with malignancy, especially T- and B-cell leukemias. These studies are directed toward defining the factors involved in the control of the human immune response. Major efforts in this area are directed toward: 1) studies of the arrangement of immunoglobulin genes and the genes encoding the antigen-specific receptor on T cells and the gene rearrangements and deletions that are involved in the control of immunoglobulin synthesis; one focus in this area is the study of new transforming genes that translocate into the immunoglobulin gene locus in certain B-cell neoplasms; 2) the genetic control of the immune response, especially as related to immune response genes associated with the major histocompatibility complex; 3) identification, purification, and molecular genetic analysis of unique cell surface determinants, especially receptors for growth factors on subpopulations of lymphoid cells with different functional capabilities using antibodies developed with hybridoma technology; 4) analysis of action of immunoregulatory cells including helper T cells, suppressor T cells, and macrophages that regulate antibody responses and on studies of disorders of these immunoregulatory cell interactions and of leukemias of these immunoregulatory cells; 5) studies of the immune response including generation of specific antibodies and cytotoxic cells to viruses; and 6) isolation and characterization of biological modifiers that regulate the human immune response. The second major goal of the Metabolism Branch is to determine the physiological and biochemical effects that a tumor produces on the metabolism of the host. Both patients with neoplastic disease as well as those with nonneoplastic disorders that facilitate the development of techniques for the study of cell membranes, homeostatic mechanisms, and metabolic dearrangements of biochemical control mechanisms are being investigated. Within this area, special emphasis is placed on the biochemical events accompanying normal growth and hormonal control of this growth as it relates to our understanding of malignant growth and on the regulatory role on intermediary metabolism played by amino acids.

MOLECULAR ANALYSIS OF REARRANGEMENT OF IMMUNOGLOBULIN GENES AND GENES ENCODING THE ANTIGEN-SPECIFIC T-CELL RECEPTOR: CLONAL AND LINEAGE MARKERS OF VALUE IN DIAGNOSIS OF LYMPHOID NEOPLASIA.

The human immunoglobulin light chain genes in their embryonic or germline state are organized in a discontinuous system of multiple germline variable (V_L) regions, alternative joining (J_L) segments, and a single or even multiple constant (C_L) regions. Heavy chains are similarly organized but have an additional (D_H) segment incorporated between the V_H and J_H regions. Human immunoglobulin (Ig) genes rearrange in an ordered fashion as pluripotent stem cells mature into immunoglobulin-synthesizing B cells and plasma cells.

During pre-B-cell development, heavy chain genes rearrange before light, and κ light chain genes generally precede λ. Dr. Korsmeyer demonstrated that this ordered process includes an unanticipated deletion of the constant κ (C_κ) and κ enhancer gene sequences that precede λ gene rearrangement. Seventy-five percent of the time this deletional recombination was located 3' to the joining (J_κ) segments. Dr. Korsmeyer cloned the recombinational element responsible for this deletion from three separate alleles and found them to be identical. This κ-deleting element, or Kde, recombined site specifically with a palindromic heptamer signal (CACAGTG) located in the J_κ-C_κ intron. All loses of C_κ genes in other pre-B cells were mediated by this Kde. This included the 25% of instances when this element recombined with sequences 5' to J_κ. In contrast, the Kde remained in its germline form on all successful κ-producing alleles. The Kde and its gene deletion are evolutionarily conserved as heteroduplex, and sequence comparisons reveal a high homology of this element between mouse and man. These data argue that the κ-deleting element defined by Dr. Korsmeyer and his associates helps ensure isotypic and allelic exclusion of light chains and could mediate the ordered use of human light chain genes. The ordered sequence of gene rearrangements was initially discovered by examining the non-T, non-B form of acute lymphoblastic leukemia (ALL) which proved to be a developmental spectrum of pre-B cells. Dr. Korsmeyer exploited the Ig gene rearrangements as tumor-specific clonal markers to follow the natural history of ALL. He observed clonal evolution from initial diagnosis to relapse which represented genetic progression, such as DJ $\longrightarrow$ VDJ rearrangements, heavy chain to heavy chain plus light chain rearrangements, and heavy chain deletions. Moreover, he has used the sensitivity of this approach to identify clonal populations of cells in bone marrow during clinical remission in some patients with ALL a full 6 and 9 months prior to histopathologic evidence for relapse. Patients with B-cell follicular lymphomas frequently manifest t(14;18)(q32;q21) translocations. Dr. Korsmeyer searched for unexpected rearrangements of the Ig heavy chain gene locus within such t(14;18)-bearing human B-cell lymphomas. He cloned the chromosomal breakpoint from the SU-DHL-6 cell line in which chromosome segment 18q21 was introduced flush with the J_{H6} joining region. This suggests that the same site-specific recombinatorial machinery which mediates DJ joining also mediates this interchromosomal translocation. The 18q21 element isolated mediates translocations in all four t(14;18)-bearing cell lines studied and in six of 11 unselected follicular lymphomas, but did not normally rearrange in other B or non-B cells. In eight of 10 cases the chromosomal breakpoints clustered within a small 4.3-kb region on chromosome 18. The breakpoints on chromosome 14 were also focused within or immediately 5' to J_H. Significantly, this location of breakpoints uniformly retains the Ig enhancer region in close proximity to the new transcriptional unit identified on chromosome segment 18q21. Regions of the genomic clone he isolated from 18q21 recognize a 6.5- and a 4.0-kb message within t(14;18)-bearing lymphoma cells. Dr. Korsmeyer and coworkers cloned the cDNAs corresponding to these transcripts and showed that they have no close homology to known v-onc, c-onc genes. Therefore, cloning this element provided the opportunity to characterize a potentially new transforming gene which, when introduced into the Ig heavy chain locus, may play a critical role in the altered growth or differentiation of the t(14;18) follicular lymphomas.

Previously, Drs. Korsmeyer and Waldmann used recombinant DNA technology involving analysis of Ig gene rearrangements to classify neoplasms previously of uncertain lineage, to aid in the diagnosis of neoplasms of the B-cell series and to define the state of differentiation of neoplastic B-cell precursors.

Over the past year, Dr. Waldmann has turned to a molecular genetic analysis of the genes encoding the T-cell antigen receptor to provide an analogous marker for T-cell clonality and lineage. The human antigen-specific T-cell receptor has been shown to be a polymorphic disulfide-linked heterodimer with a M_r of approximately 90,000 daltons consisting of an approximately 45,000 to 50,000 dalton α subunit and a 40,000 to 45,000 dalton β subunit. cDNA clones encoding the α and β chains of the T-cell receptor had been isolated by Davis and Mak and their coworkers. The human T_β chain gene in its germline form is composed of discontinuous gene subsegments consisting of multiple variable regions (V_β), with duplicate sets of diversity ($D_{\beta 1}$, $D_{\beta 2}$), joining ($J_{\beta 1}$, $J_{\beta 2}$), and constant ($C_{\beta 1}$, $C_{\beta 2}$) gene segments. At some point during the differentiation of a pluripotent stem cell into a mature T cell, a process of DNA rearrangement juxtaposes one D_β with a J_β segment and then a V_β region with that D_β/J_β juncture to assemble the complete T_β variable region gene. Dr. Waldmann utilized a cDNA clone that recognizes both the $C_{\beta 1}$ and $C_{\beta 2}$ gene complex to determine the arrangement of the T_β chain gene in human lymphoid neoplasms. The analysis of rearrangements of the T-cell antigen receptor gene proved to be of value in the classification of neoplasms of uncertain lineage. Furthermore, these studies have culminated in the development of new approaches for the diagnosis and monitoring of the therapy of T-cell lymphoid neoplasia. In initial studies, the germline arrangement of the T_β chain gene was defined by examining the DNA from circulating white blood cells of normal individuals using the ^{32}P-labeled cDNA clone that hybridizes to the $C_{\beta 1}$ and $C_{\beta 2}$ gene segments of this gene. All neoplastic expansions of T cells or their precursors, including those from patients with the helper Sézary leukemia, those from patients with the suppressor human T-lymphotrophic retrovirus (HTLV-I)-associated adult T-cell leukemia (ATL), and those from patients with acute lymphocytic leukemia that expressed T-cell-associated antigens, displayed an identifiable DNA rearrangement. In virtually all cases the leukemic cells manifested multiple rearrangements identifiable as two new bands on Southern analysis of DNA from the malignant tissue not present in germline DNA. In contrast to the rearrangement in T cells, the majority of clonal B cells, as well as malignancies of nonlymphoid series, did not display rearrangement of the T_β gene. Normal polyclonal T cells from peripheral blood presented a pattern distinct from that of germline tissues or clonal leukemic T cells. Normal polyclonal T cells possess numerous different T_β gene rearrangements. Collectively, none of these gene rearrangements is detectable as a new band on a Southern blot because they are below the threshold of sensitivity of this method. However, on Southern analysis, such polyclonal T cells have a marked dimunition in the intensity of the germline 11-kb EcoRI band when compared to the 4-kb band identified by the T_β probe. Thus, polyclonal T cells, clonal T cells, and non-T cells can be distinguished from each other on the basis of their arrangement of the T_β gene as assessed by Southern analysis. Southern blot analysis of clonally rearranged T_β genes has sufficient sensitivity to detect even minority (2-5%) populations of clonal cells within tissues admixed with normal cellular elements. There are few technqiues available to define clonality in T-cell populations. Dr. Waldmann recently addressed a controversial issue concerning the clonality of the circulating lymphocytes in the syndrome characterized by lymphocytosis of large granular lymphocytes expressing the T3 and T8 antigens associated with granulocytopenia and anemia. It is uncertain on the basis of previous studies whether this disorder represents a slowly growing chronic lymphocytic leukemia of T cells or merely an expansion of a polyclonal immunoregulatory T-cell population. In three of the four patients studied, the arrangement of the T_β genes in the peripheral

blood mononuclear cells of patients with such lymphocytosis and granulocytopenia, there was a classical clonal pattern of the T_β chain gene rearrangements. Thus, in these patients, the T8 lymphocytosis associated with granulocytopenia appears to be an expansion of a single clone of lymphocytes of the T8 subset. As discussed below, an analysis of the T_β gene arrangement has been of value as an adjunct in monitoring the therapy of patients with leukemias of the T-cell series. In a patient with ATL expressing the Tac antigen, response to therapy was associated with a loss of the new band on Southern gel that is the hallmark of the clonal population. At a time before clinical relapse was observed, clonal cells with the rearranged band were again demonstrable with a C_β probe. Within 2 months leukemic cells were again morphologically identifiable in the peripheral blood and there was a clinically evident recurrence of the T-cell malignancy. During the final relapse, evidence of clonal progression was obtained since a new rearrangement of the T_β gene was observed in the dominant leukemic cell populations. This was paralleled by a failure of response of the patient to anti-Tac immunotherapy, a therapy which was effective previously. Thus, in initial studies, the use of the Ig gene rearrangements was of great value in the study of B lymphocytes; however, their application was predominantly restricted to cells of the B-cell lineage. In the recent studies, Dr. Waldmann demonstrated that T-cell receptor rearrangements, taken in conjunction with studies of Ig gene rearrangements, aid in the definition of the lineage (T-cell versus B-cell) and the clonality of lymphoid populations of all series. The application of this molecular genetic approach has great potential for complementing conventional marker analysis, cytogenetics, and histopathology, thereby broadening the scientific basis for the classification, diagnosis, and monitoring of the therapy of lymphoid neoplasia.

THE GENETIC CONTROL OF THE IMMUNE RESPONSE

A primary goal of Dr. Berzofsky's group has been to understand, and ultimately manipulate, the immune response at the antigen-specific level. They have been approaching this problem through studies of immune response (Ir) genes, of the structures of antigenic proteins required for activation of T lymphocytes, of the interaction between monoclonal antibodies and defined sites on protein molecules, and of the regulatory networks of antibodies recognizing specific combining site structures of these antibodies (idiotypes). These studies have also led to the discovery of two approaches to enhance or amplify weak antibody responses (immunopotentiation). Earlier work from Dr. Berzofsky's laboratory using sperm whale myoglobin as a model antigen demonstrated that there were two antigenic sites that were immunodominant for T-lymphocyte responses of a high responder strain of mice; that is, most of the T lymphocytes responding to myoglobin were specific for one or the other of these sites. Moreover, each site was seen by T cells only in association with a particular major histocompatibility molecule ($I\text{-}A^d$ or $I\text{-}E^d$) on the surface of another cell, an antigen-presenting cell. Thus, for both T-cell clones or bulk T-cell populations when only $I\text{-}A^d$ was available, the site around glutamic acid 109 was immunodominant, whereas when only $I\text{-}E^d$ was available, the site around lysine 140 was immunodominant. Thus, immunodominance depends strongly on the major histocompatibility molecule with which the antigen is presented. Using peptides synthesized corresponding to these two sites, the peptide consisting of residues 132-146 was fully active for stimulating T-cell clones specific for the Lys 140/$I\text{-}E^d$ site, and the peptide 102-118 was fully active for stimulating T-cell clones specific for the Glu 109/$I\text{-}A^d$ site. Peptide variants of the former site have

shown: 1) that the minimum peptide which stimulates is 136-146, so that these 11 residues have all the information necessary to stimulate a T cell, including not only the site binding to the T-cell receptor, but also any site which may be necessary to interact with $I\text{-}E^d$; and 2) that four residues contribute to the activity. These are all hydrophilic residues spaced approximately every turn of the alpha helix, so that they all appear on the same face of the helix in the native structure. This face is exposed to water in native myoglobin. However, Dr. Berzofsky has also found that the native myoglobin cannot stimulate these T cells unless it is first cleaved or unfolded to expose the other, hydrophobic side of the helix which is buried in the interior of the native protein. Thus, both faces of the alpha helix are necessary for activity. The segregation of hydrophilic and hydrophobic structures to different faces of the helix define 132-146 as an amphipathic alpha helix. The segment of residues 102-118 is also an amphipathic alpha helix in native myoglobin. The finding that both of the known immunodominant T-cell sites of myoglobin are amphipathic led Dr. Berzofsky to ask, in collaboration with Dr. Charles DeLisi, whether this was a general property of sites antigenic for T cells. Since T cells generally recognize globular proteins only after they have been cleaved into fragments by the antigen-presenting cell, the tertiary structure of the native protein should not be important for activity. Thus, it may be possible to predict T-cell antigenic sites on the basis of sequence and secondary structure alone more easily than sites binding to antibodies. To test this hypothesis, they determined the most intense periodicity of hydrophobic residues for overlapping seven-residue segments along the sequence of six proteins for which a total of 12 sites fell in regions with hydrophobic periodicity corresponding to that of an amphipathic alpha helix, with a statistical significance for each protein of <0.01. Thus, T-cell antigenic sites were shown to be amphipathic structures, especially amphipathic alpha helices. Although Dr. Berzofsky has not yet shown the converse, that most amphipathic helices are good T-cell antigenic sites, he is currently exploring this prospect as a potentially powerful predictive tool for locating T-cell antigenic sites which may be very useful for designing synthetic vaccines.

Dr. Berzofsky's recent studies have led to the discovery of two new methods of immunopotentiation which may be useful for vaccines. First, he found that incorporation of interleukin-2 (IL-2) in the adjuvant with the antigen at the time of immunization enhanced the antibody response of genetically low responders 100-fold to the level of high responder mice. The mechanism may involve amplification of low levels of T-cell help. This approach may be useful for immunization of immunodeficient patients. In a second approach, studies of uptake and presentation of antigen led to the idea of targeting the antigen to the immune system by coupling the antigen to anti-Ig which can bind to B lymphocytes. Dr. Berzofsky compared the potency for T-cell stimulation *in vitro* and the immunogenicity for antibody production *in vivo* of free ferritin and ferritin coupled to goat anti-mouse IgG, anti-IgM, or anti-IgA. He found that the ferritin coupled to anti-IgG or to anti-IgM stimulated ferritin-specific T cells at a 10- to 100-fold lower concentration than free ferritin and was effective with fewer antigen-presenting cells. The enhancement in antigenic potency depended on the uptake and presentation by B lymphocytes. Similarly, *in vivo*, the ferritin-anti-IgG conjugate was active at a 50-fold lower dose for immunization than control antigens in inducing serum antibody production. This approach of targeting the antigen to the immune system to enhance immunogenicity may make possible successful immunization with very weak antigens or antigens available in only small quantities.

LYMPHOCYTE SURFACE RECEPTORS FOR GROWTH FACTORS AND LYMPHOKINES

A major accomplishment of the Metabolism Branch over the past year by Drs. Greene and Waldmann and their coworkers has been the definition of T-cell growth factor receptors and the molecular cloning and characterization of these receptors in normal and malignant T cells. Using hybridoma technology, they have developed a monoclonal antibody, termed anti-Tac, that identifies the T-cell growth factor receptor and that blocks the interaction of IL-2 with its receptor. Using this antibody, cDNAs encoding the human receptor for IL-2 have been molecularly cloned, sequenced, and expressed in eukaryotic cells by Dr. Greene and coworkers. The mature receptor protein is composed of 251 amino acids which include two N-glycosylation sites, 13 cysteine residues available for intrachain disulfide bonding, multiple potential O-linked glycosylation sites, a 19-residue hydrophobic transmembrane domain, and a short, positively charged 13-amino-acid intracytoplasmic domain. Sequencing analysis of overlapping genomic phage clones isolated by Dr. Greene's group has revealed that the normal IL-2 receptor (IL-2R) is encoded by eight separate exons spanning more than 25 kb of DNA. In situ hybridization has localized the IL-2R gene to the short arm of chromosome 10 (band p14-15). The boundaries of these exons correspond to peptide domains within the receptor protein. Exons 2 and 3 share considerable homology with exons 4 and 5, suggesting a remote gene duplication event and raising the possibility that a single receptor molecule may contain two IL-2 binding sites. Transcription of the IL-2R gene involves the use of two discrete promoters in normal activated T cells located 60 bases apart immediately 5' to exon 1. Post-transcriptional splicing of IL-2R mRNA may infrequently result in removal of the 216-bp segment corresponding to exon 4. This aberrant splicing apparently results in a protein which is unable to bind either IL-2 or anti-Tac. Use of at least three different polyadenylation signal sequences results in the production of different size mRNA species (1550 and 3500 bases). The normal rise and fall in IL-2R expression, which contributes to the regulation of normal T-cell activation, is controlled at the level of gene transcription. Temporally, transcription of the IL-2R gene occurs prior to IL-2 gene activation. Senescent activated T cells, which have lost the majority of their IL-2Rs and do not actively proliferate, can be activated to reexpress large numbers of IL-2Rs and proliferate by exposure to mitogenic lectins, agents which activate protein kinase C (PMA, phospholipase C, and diacylglycerol), or IL-2. Each of these agents produces transciptional activation of the IL-2R gene. The primary translation product of the IL-2R was shown by Dr. Greene to have a M_r of 34,500. Removal of the 21-amino-acid signal peptide generates the polypeptide backbone of the receptor which has a M_r of 33,000. This protein is cotranslationally modified by N-glycosylation yielding two intermediate precursors (M_r 35,000 and 37,000). These precursors are exported to the Golgi apparatus where O-linked sugar, sialic acid, and sulfate are added. Mature receptor protein with a M_r of 55,000 is displayed on the cell surface and may exist in either high or low affinity states. The growth-promoting effects of IL-2 appear to be mediated by the high affinity IL-2Rs, while the function of the low affinity receptors is unknown. The molecular and biochemical differences of high and low affinity receptors are unresolved; however, the high affinity receptors may involve the formation of a receptor complex at the plasma membrane. Only the high affinity form of the receptor undergoes receptor-mediated endocytosis following ligand binding.

Utilizing anti-Tac, Drs. Waldmann and Greene have defined those reactions that require an interaction of IL-2 with its inducible receptor. Previously, they had found that anti-Tac blocks T-cell proliferation induced by antigens, abrogates the generation of cytotoxic T lymphocytes but does not inhibit their action once generated, and inhibits the sequential development of late-appearing activation antigens on T cells. Over the past year Dr. Waldmann and associates have shown that anti-Tac inhibits concanavalin A, Epstein-Barr virus, and high antigen-induced suppressor T-cell generation. Previously, they had shown that anti-Tac inhibited Ig production, while B cells were activated by pokeweed mitogen, wheat germ agglutinin, streptomycin 0, and Nocardia water-soluble mitogen. Recently, Dr. Waldmann has shown that the specific antibody response to sheep red blood cells, rickettsia antigens, and haemophilus influenza antigens in vitro is inhibited by anti-Tac. Furthermore, he has shown that normal B cells could be activated to express IL-2Rs, to produce mRNA encoding the IL-2R, and to proliferate on addition of purified IL-2. Furthermore, B cells, T cells, and other lymphocyte populations were shown to manifest upregulation of IL-2Rs on addition of recombinant IL-2 to cells expressing small numbers of such receptors. This upregulation of IL-2Rs required protein synthesis and RNA transcription but not cell division.

Dr. Nelson and his coworkers have recently characterized a monoclonal antibody, 7G7/B6, which binds to the human IL-2R at a site distinct from its ligand, IL-2, and anti-Tac. Using the two available monoclonal antibodies, Dr. Nelson has developed a "sandwich" ELISA for the quantitative measurement of IL-2R in solution. Using this assay, Dr. Nelson has been able to measure soluble IL-2R in detergent-solubilized extracts of activated but not resting normal cells. In addition, he made the discovery that a soluble form of the IL-2R is released into the cell-free culture supernatant of activated normal T cells and B cells in vitro. The release of this soluble IL-2R could be induced by plant lectins, soluble and cell-associated alloantigens, monoclonal antibodies against the T-cell antigen receptor complex, and by IL-2. In addition to the in vitro release of IL-2R by activated normal lymphocytes in vitro, Dr. Nelson also found soluble IL-2R constitutively released from certain tumor cell lines, particularly those infected with HTLV-I and those transformed with the Epstein-Barr virus. In further studies the released soluble IL-2R was shown to have a M_r of 40,000 to 45,000 daltons, approximately 10,000 daltons smaller than the cell-associated receptor, and that the released IL-2R is capable of binding its ligand, IL-2. Thus, the released, soluble form of the IL-2R may play an immunoregulatory role by competing with cell-associated IL-2R for available ligand, thus downregulating cell growth and maturation. Dr. Nelson has also examined the in vivo counterpart of these observations by measuring the levels of serum IL-2R in a variety of conditions. The serum of normal individuals contained low but measurable serum levels of IL-2R (mean 186 U/ml). When the serum of various patients with cancer was studied, patients with the HTLV-I-associated ATL had mean serum levels of IL-2R in the range of 20,000-30,000 U/ml, a value significantly greater than normal. In addition, patients with the Sézary syndrome, Hodgkin's disease, chronic lymphocytic leukemia, certain solid organ malignancies, but not multiple myeloma, had serum IL-2R levels significantly greater than normal. The elevated serum levels of IL-2R in certain patients with cancer may inhibit the host's immune response to the tumor and thereby allow tumor growth. In addition, there were increases in the serum IL-2R following the administration of recombinant DNA-derived IL-2 to human cancer patients in vivo. The appearance of this soluble receptor in the serum of such

treated patients may limit the usefulness of this form of immunotherapy. More recently, Dr. Nelson found significant elevations of serum IL-2R in the sera of patients infected with another human lymphotropic retrovirus, HTLV-III/LAV, including those with the acquired immune deficiency syndrome (AIDS), those with the lymphadenopathy syndrome, those with the AIDS-related complex, and asymptomatic sexual partners of affected individuals. This observation may be related to the immune dysfunction observed in these patients. The discovery of a released soluble form of IL-2R in the serum of normal individuals and abnormalities of the level of IL-2R in various disease conditions may provide a sensitive diagnostic test and a means of following therapy in patients with abnormalities of immune activation in vivo.

As noted above, ATL is produced by HTLV-I retroviral infection of human T cells. ATL leukemic cell lines uniformly express large numbers of IL-2Rs which occasionally may be aberrant in size. This increase in IL-2R number is not the result of receptor gene rearrangement or amplification, nor is it caused by chromosome 10 translocations involving the IL-2R. ATL T cells constitutively transcribe IL-2R mRNA but, paradoxically, receptor transcription was inhibited by PHA and PMA, agents which activate IL-2R gene transcription in normal T cells. Primer extension in S1 nuclease assays by Dr. Greene's group suggested a third normally cryptic promoter may be utilized in these leukemic cells. In further studies the LOR protein encoded in part by the pX region of the HTLV-I virus may act as a trans-acting, transcriptional activator that induces IL-2R gene expression, thus providing an explanation for the constant association of IL-2R expression in HTLV-I-infected lymphoid cells. The constant expression of large numbers of IL-2Rs which may be aberrant may play a role in the uncontrolled growth of ATL leukemic cells. Dr. Waldmann has initiated a clinical trial to evaluate the efficacy of intravenously administered anti-Tac monoclonal antibody in the treatment of patients with ATL. The scientific basis for these studies is the observation that ATL leukemic cells express the Tac antigen, whereas normal resting T cells and their precursors do not. Three patients with ATL have been treated with intravenously administered anti-Tac. None of the patients suffered any untoward reaction nor did they produce antibodies reactive with mouse Ig or the idiotype of the anti-Tac monoclonal. Furthermore, there was no decline in any of the normal formed elements of the blood. Two patients with rapidly developing forms of ATL had transient responses; however, therapy of the other patient was followed by a 6-month remission as assessed by regression of skin lesions, by routine hematological tests, by immunofluorescence analysis, and by molecular genetic analysis of the arrangement of the T-cell receptor beta chain genes. When the patient ultimately relapsed developing circulating leukemic cells and large malignant skin lesions, a second course of intravenous infusions of anti-Tac was followed by the virtual disappearance of skin lesions and an over 90% reduction in the number of circulating cells. Three months subsequently, the leukemic cells were again demonstrable in the circulation. At this time the leukemia was no longer responsive to infusions of anti-Tac and the patient required further chemotherapy. At the time of the second relapse, the patient's leukemic cells had undergone clonal progression. They were shown to be from the same clonal origin, on the basis of an identical site of integration of the HTLV-I virus. However, they had undergone a further rearrangement of the T_β genes as assessed using molecular probes to the T_β constant region.

The therapeutic studies have been extended in vitro by examining the efficacies of toxins and isotopes coupled to anti-Tac in selectively inhibiting protein synthesis and the viability of Tac-positive ATL leukemic cell lines. The addition of anti-Tac coupled to the A chain of the toxin ricin or to the α-emitting isotope of bismuth (^{212}Bi-anti-Tac) effectively inhibited protein synthesis by the HTLV-I-associated Tac-positive ATL line HUT 102-B2. In contrast, conjugates of ricin A or of radiolabeled bismuth to a control monoclonal of the same isotype did not inhibit protein synthesis when used in the same concentration. Higher concentrations of the toxin or isotopic conjugates did inhibit the proliferation of Tac-negative T cells. In parallel studies performed in collaboration with Drs. David FitzGerald and Ira Pastan, Pseudomonas exotoxin conjugates of anti-Tac inhibited the protein synthesis by HUT 102-B2 cells, but not that of the acute T-cell leukemia line Molt-4 that does not express the Tac antigen. The toxicity of the anti-Tac toxin conjugates could be inhibited by adding excess unlabeled anti-Tac. Pseudomonas exotoxin conjugates of anti-Tac showed only minimum toxicity in cylomologus monkeys. Thus, the development of Pseudomonas exotoxin conjugates of the monoclonal anti-Tac that are directed toward the IL-2R expressed on adult T-cell leukemic cells may permit the development of a rational approach for the treatment of this almost uniformly fatal form of leukemia.

IMMUNOREGULATORY INTERACTIONS IN THE IMMUNE RESPONSE

Dr. Blaese and his associates have had a long-term interest in the effects of the herpes group of viruses on the immune response in humans. In addition to their extensive and continuing investigation of the effects of the Epstein-Barr virus on human B-cell differentiation and the immunoregulatory T-cell mechanisms which control this virus, they have recently evaluated the nature of the response to cytomegalovirus (CMV). CMV is an opportunistic herpes virus which infects the majority of people by adult life, but which is associated with disease primarily in the neonate and in immunocompromised hosts. CMV presents a major problem in patients receiving organ or bone marrow transplantation and in patients with AIDS. Dr. Blaese found that lymphocytes in culture respond to stimulation with CMV with impressive T-cell proliferation accompanied by B-cell activation and Ig synthesis. Ig production induced by CMV is polyclonal, induces all isotypes, and often equals the response induced by pokeweed mitogen or Epstein-Barr virus. In contrast to Epstein-Barr virus, however, CMV induced Ig production only in cultures containing viable, unirradiated T cells and further, only in lymphocyte donors who were CMV immune. Ultraviolet irradiation or heat-killed CMV was as effective as infectious virus in inducing these responses, again in contrast to Epstein-Barr virus where only viable infectious virus is stimulatory. These experiments indicate that CMV antigens are potent T-cell-dependent polyclonal B-cell activators and may help to explain some of the striking polyclonal B-cell activation and hypergammaglobulinemia commonly seen in patients with congenital or acquired CMV infections.

ISOLATION AND CHARACTERIZATION OF BIOLOGICAL MODIFIERS THAT REGULATE THE HUMAN IMMUNE RESPONSE

Dr. Muchmore's laboratory has two major interrelated areas of research interest. The first project concerns the purification and characterization of immunoregulatory factors associated with human pregnancy. This has resulted recently in the purification to apparent homogeneity of a previously undescribed suppressor

immunoregulatory glycoprotein which he terms uromodulin. Uromodulin acts at picomolar concentration and appears to specifically block the in vitro activation of interleukin-1 (IL-1). Uromodulin is in the process of further molecular characterization and has theoretical importance not only for pregnancy but also for its potential role as a regulator of normal immune responses. The second area of investigation concerns the hypothesis that sugar sequences associated with glycoproteins and glycolipids may function as receptors for endogenous lectins. Dr. Muchmore has done extensive characterization of the in vitro immunoregulatory role of monosaccharides and is now concentrating on complex oligosaccharides. He has isolated two candidate oligosaccharides with marked in vitro immunosuppressive activity. These compounds are from five to six orders of magnitude more active than corresponding monosaccharides. These complex oligomers are of great theoretical interest, but more importantly, they suggest a new class of immunosuppressive drugs.

MECHANISM OF ACTION OF INSULIN-LIKE GROWTH FACTORS

Dr. Peter Nissley has been studying the mechanism of action of insulin-like growth factors (IGFs), emphasizing an analysis of the cellular receptors for these hormones. IGF-I and -II are closely related to insulin by amino acid sequence data and more recently by base sequencing of genomic DNA. In addition to an in vivo role for IGF-I as the mediator of pituitary growth hormone action, the IGFs are mitogens for a variety of cells in culture. There are two cell surface receptors for the IGFs. The type I receptor binds IGF-I better than IGF-II and recognizes insulin weakly. This receptor is very similar to the insulin receptor structurally. The type II receptor prefers IGF-II over IGF-I and does not recognize insulin; it is structurally quite distinct from the type I receptor. Dr. Nissley has been developing antibodies to the type II receptor which should be useful in sorting out the relative importance of the type I and type II IGF receptors in mediating particular biological responses associated with growth. Most of these efforts have been directed toward developing antibodies against a rat type II receptor derived from the Swarm chondrosarcoma. Dr. Nissley previously had purified this type II receptor to homogeneity. Both blocking and immunoprecipitating antibodies were detected in the sera of mice immunized with rat type II receptor preparations.

Like the insulin receptor, the type I receptor has been shown to have intrinsic tyrosine kinase activity directed toward the β subunit and extrinsic tyrosine-containing substrates. Similar tyrosine kinase activity has been associated with the transforming proteins of retroviruses. It was of interest, therefore, to see whether or not the type II receptor displayed similar properties. Dr. Nissley has been unable to demonstrate tyrosine kinase activity in highly purified preparations of the type II receptor, either directed toward the receptor itself or against extrinsic tyrosine-containing substrate. By contrast, the type II receptor is phosphorylated in intact cells, and this phosphorylation is dependent upon IGF-II in a cell line which does not produce IGF-II. It remains to be shown whether this phosphorylation plays a role in receptor regulation or function.

Dr. Nissley previously showed that rat embryo fibroblasts produced IGF-II; production of IGF-I was low or absent. By contrast, postnatal fibroblasts produced IGF-I but not IGF-II. This developmental pattern of IGF production mimicked the developmental pattern of serum levels of IGF-I and -II in the rat. Conse-

quently, Dr. Nissley asked whether a similar production pattern was true for human fetal and postnatal fibroblasts. Dr. Nissley found that IGF production was much lower in human fibroblasts and that the pattern of IGF-I and IGF-II production was similar in fetal and postnatal fibroblasts. He found that the human fibroblasts were producing binding proteins for IGFs, probably identical to the binding proteins that he found in serum. These binding proteins complicated the interpretation of receptor binding studies on human fibroblast monolayers.

REGULATORY FUNCTIONS OF PROLINE AND ITS METABOLITE, PYRROLINE-5-CARBOXYLATE

Dr. Phang previously showed that the synthesis of phosphoribosyl pyrophosphate (PRPP) and ribonucleotides is stimulated by pyrroline-5-carboxylate (P5C). This intermediate in the interconversions of proline, ornithine, and glutamate can activate the pentose phosphate pathway by a redox-dependent mechanism, thereby serving as a metabolic interlink between amino acids and ribonucleotides. Dr. Phang focused on the physiological effects of this newly discovered regulatory mechanism. First, he showed an interaction between the P5C-mediated mechanism and the effect of growth factors on ribonucleotide reductase. Second, he identified P5C as a circulating molecule in human plasma and showed that the levels of this intercellular communicator are sensitive to dietary perturbation. There are two response patterns in the interaction between P5C and growth factors. The response to a progression factor--multiplication stimulation activity (MSA), a rat IGF-II--differed from that to a competence factor, platelet-derived growth factor (PDGF). Using purified MSA, the effect of P5C was additive to that of MSA on PRPP levels. In marked contrast, the effect of P5C increased synergistically as a function of PDGF concentration. At saturating concentrations of PDGF, the addition of P5C markedly increased the levels of PRPP over that of PDGF alone. Treatment with PDGF increased the P5C effect 8- to 10-fold over its effect in quiescent cells. Even at concentrations of PDGF which are markedly inadequate to stimulate PRPP, the effect of P5C is markedly augmented. These patterns of response suggest that P5C and PDGF stimulate PRPP by different mechanisms, but that P5C and MSA may work by similar mechanisms. If the P5C-mediated mechanism contributes to the MSA effect on PRPP synthesis, one would expect a deficient response to MSA in a cell line unable to synthesize P5C. Using a clone of Chinese hamster ovary cells which lack the enzymes of P5C synthesis, Dr. Phang found this was indeed the case. The effect of MSA on PRPP was blunted in this cell line. More importantly, the response was restored to normal with added P5C. Thus, endogenously synthesized P5C may mediate, at least in part, the stimulation of PRPP synthesis by MSA. Since the synergistic stimulation of PRPP by PDGF and P5C suggested that they work through different mechanisms, Dr. Phang examined whether they might differ in their sensitivity to cycloheximide, an inhibitor of protein synthesis. Cells were incubated with 0.5 mM cycloheximide for 30 min before the addition of P5C or PDGF. Under these conditions, protein synthesis was inhibited by 85%. Dr. Phang found that cycloheximide had no effect on either control or P5C-stimulated levels of PRPP in the absence of growth factors. The effect of PDGF, however, was markedly decreased by cycloheximide. The augmentation of the P5C effect by PDGF, interestingly, was not inhibited by cycloheximide. Thus, although the stimulatory effect of PRPP by PDGF requires either a newly synthesized or a short half-lived protein, the PDGF-mediated augmentation of the P5C effect on PRPP does not.

The *in vitro* interaction between growth factors and added P5C would be physiologically relevant if P5C is a circulating intercellular communicator. Indeed, this is the case. In normal human volunteers, the fasting level of P5C in venous plasma is 0.40 $\pm$ 0.12 μM. Of special interest, the level can increase 10- to 15-fold with dietary perturbation. Frequent blood samples were obtained for P5C levels following a controlled isocaloric intake of carbohydrates, protein, and fats. Accompanying a meal high in protein, the levels of P5C can rise to 3-5 micromolar, concentrations which can have marked effects on PRPP levels in intact cells *in vitro*. This finding, together with the observed *in vitro* synergism between P5C and growth factors, may provide an approach for understanding the nutritional effects of high protein intake on tumor initiation and promotion.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04002-16 MET

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Defects in Immunoregulatory Cell Interactions in Patients with Immune Dysfunction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Thomas A. Waldmann	Branch Chief	MET,NCI
	Stanley J. Korsmeyer	Senior Investigator	MET,NCI
	Warner C. Greene	Senior Investigator	MET,NCI
	Warren Leonard	Senior Staff Fellow	MET,NCI
	Monica Peacock	Medical Staff Fellow	MET,NCI
	Robert Kozak	Guest Worker	MET,NCI
	Tsutomu Oh-Ishi	Visiting Fellow	MET,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	4	2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The rearrangements of immunoglobulin and T-cell antigen receptor genes were analyzed to develop approaches for the classification of neoplasms of uncertain lineage, to define the state of differentiation of neoplastic B-cell and T-cell precursors, and to determine the mechanisms that lead to the failure of maturation of such precursor cells. These studies have culminated in the development of new approaches for the diagnosis and monitoring the therapy of T- and B-cell lymphoid neoplasia. Furthermore, studies were directed toward defining the role played by the inducible receptor for interleukin-2 (IL-2) in the proliferation of normal and malignant T lymphocytes. Using a monoclonal antibody (anti-Tac) to the IL-2 receptor, this receptor was purified, biochemically characterized, and the cDNAs hybridizing with it were molecularly cloned, sequenced, and expressed. In contrast to normal resting T cells, human T-cell lymphotrophic virus-I (HTLV-I)-associated adult T-cell leukemia (ATL) cells constitutively express large numbers of IL-2 receptors which may be aberrant. The anti-Tac monoclonal antibody was used in the therapy of the now universally fatal, Tac-expressing ATL. One of three patients treated underwent a complete remission lasting 6 months. In additional studies, ricin A, Pseudomonas exotoxin, and the alpha-emitting isotope of bismuth were conjugated to the anti-Tac monoclonal antibody. These antibody conjugates effectively killed Tac-expressing tumor lines at concentrations that did not affect the viability of Tac-nonexpressing T lymphocytes.

Project Description

Objectives:

The objectives of the study were: to use a molecular genetic analysis of the arrangement of immunoglobulin and T-cell receptor genes to classify neoplasms of uncertain lineage, to define the state of differentiation of lymphoid tumors, to determine the mechanisms that lead to the failure of maturation of such precursor cells, and to develop new approaches for the diagnosis and monitoring the therapy of lymphoid neoplasia. Other objectives were to purify and characterize the membrane receptor for T-cell growth factor or IL-2, to clone and express the gene for this receptor, to study the expression of this receptor in varying lymphoid malignancies, to determine the effect of a monoclonal antibody (anti-Tac) to the IL-2 receptor on the human immune response in vitro, and to determine the efficacy of therapy with anti-Tac monoclonal, both unmodified and conjugated with Pseudomonas toxin, in the treatment of patients with ATL. Finally, major efforts were directed toward determining the role of the regulatory network of suppressor T cells, helper T cells, and macrophages in the control of the maturation of B cells into plasma cells, and to define the disorders of these suppressor and helper interactions that underlie primary immunodeficiency, allergic, autoimmune, and malignant diseases in humans. Furthermore, a major effort was directed toward identifying, isolating, and purifying soluble factors produced by T cells that inhibit terminal maturation of B lymphocytes into immunoglobulin-secreting cells.

Methods Employed:

Recombinant DNA techniques with ^{32}P-labeled probes to the constant region genes of the β chain of the T-cell antigen receptor, to the γ chain of T cells, and to the constant κ,λ light chain and heavy chain V,D,J, and C chain genes of immunoglobulins were used to study antigen-specific T-cell receptor and immunoglobulin gene rearrangements that occur during T-cell and B-cell maturation and to analyze the arrangements of such genes in lymphoid leukemias and immunodeficiency states. Hybridoma procedures have been used to produce a monoclonal antibody to IL-2 receptors. This monoclonal antibody was used to purify the IL-2 receptor by affinity chromatography. Utilizing an oligonucleotide probe to the determined N terminal amino acid sequence of this receptor, cDNAs encoding the IL-2 receptor were cloned and expressed in COS-1 cells. In vitro culture techniques utilizing mitogen-stimulated peripheral blood lymphocytes have been developed for the study of the terminal differentiation of B lymphocytes into immunoglobulin-secreting cells and for the analysis of helper and suppressor T-cell and monocyte activity. These techniques have been applied to the analysis of helper and suppressor T-cell functions of leukemias, the analysis of the functional effects of a monoclonal antibody to the IL-2 receptor, and to the analysis, identification, and partial purification of a suppressor molecule that inhibits immunoglobulin synthesis but does not affect T-cell proliferation.

Major Findings:

Major efforts under this project over the past few years have been directed toward defining the factors that regulate the human immune response and the disorders in these regulatory events that occur in association with immunodeficiency diseases and leukemia. Over the past year, we have focused on the study of the arrangement of immunoglobulin and T-cell antigen receptor genes and the rearrangements and deletions of these genes that are involved in the control of immunoglobulin synthesis and the generation of the T-cell receptor, as well as the rearrangements that occur in neoplasms of the B- and T-cell series. Immunoglobulins responsible for individual antibodies are organized as discontinuous DNA segments in their germline form. As an uncommitted stem cell develops into an antibody-synthesizing plasma cell, rearrangements of these immunoglobulin gene segments serve to activate the genes and to generate the virtually unlimited capacity to synthesize antibodies that recognize potential antigens. We have shown that the analysis of the immunoglobulin gene structure and arrangement is of great value in the study of human lymphoid neoplasms. Recombinant DNA technology involving analysis of immunoglobulin gene arrangement has been used to classify neoplasms previously of uncertain lineage. Specifically, it has been shown that hairy cell leukemia, the lymphoid blast crisis of chronic myelogenous leukemia, and those non-T, non-B acute lymphocytic leukemia cells that do not react with monoclonals to T cells are of the B-cell and B-cell precursor series. An analysis of the B-cell precursor leukemia has been used to demonstrate that there is an ordered hierarchy in immunoglobulin gene rearrangement with heavy chain genes preceding light chain genes and κ light chain genes preceding λ genes. The approaches using immunoglobulin gene probes have been used to aid in the diagnosis of neoplasms of the B-cell series, to define recurrence of B-cell leukemias, to define the state of maturation of neoplastic B-cell precursors, and to determine the mechanisms that lead to the failure of maturation of such precursor cells.

Over the past year, we have performed a similar analysis of T-cell malignancies using cDNA clones that recognize the genes encoding the β chain of the T-cell receptor. The antigen receptor on T lymphocytes is a polymorphic heterodimer of T_α and T_β chains. Genes encoding these peptides in their germline form are separated DNA segments that are joined by recombinations during T-cell development. We have shown that the detection of such rearrangements of the T_β gene provides a sensitive marker for both clonality and T-cell lineage. We have used a molecular genetic analysis with a cDNA clone recognizing the constant region of the gene encoding the T_β chain to demonstrate that the Sézary leukemia, ATL, and acute lymphocytic leukemia cells reactive with monoclonals directed toward T cells have T_β gene rearrangements, indicating that they are clonal expansions of T cells. Furthermore, circulating T cells were shown to be clonal in five of seven patients with the T8-cell-predominant lymphocytosis associated with granulocytopenia. The T8 lymphoproliferative disease usually is not an aggressive one, although the patients are often leukopenic and have recurrent bacterial infections requiring antibiotic therapy. Most of the cell populations express antibody-dependent cellular cytotoxicity (ADCC) and in certain cases, natural killer or suppressor activities. Different scientific groups have suggested that this is either a polyclonal lymphoproliferative disease or, alter-

natively, a chronic leukemia; however, it has been emphasized that no definitive evidence for clonality has been reported. We demonstrated a clonal pattern of T_β gene rearrangement in the majority of cases; thus, this disorder is usually a clonal expansion of large granular lymphocytes expressing the T8 phenotype. Finally, as discussed more extensively below, T_β gene rearrangements were used to monitor the therapy of patients with ATL. These sensitive indicators of clonality located at the DNA level are capable of providing insights into the cellular origin, diagnosis, and natural history of T-cell neoplasia.

A third gene family (termed γ) that rearranges during somatic development of T cells has been identified in addition to the two gene families (α and β) that encode subunits of the antigen-specific receptor of the T cell. In collaboration with Jonathan Seidman, it has been demonstrated that this γ gene family is present in the human genome on the short arm of chromosome 7 (7p15-21). The γ chain genes are encoded by gene segments that are analogous to the immunoglobulin gene variable, constant, and joining segments. There are two closely related constant region gene segments in the human genome. One of the constant region genes is involved in rearrangements and deletions in all T-cell leukemias studied. In contrast, the B cells examined did not show rearrangement or deletions of the γ chain genes. T-cell lines derived from patients with the immunodeficiency syndrome ataxia telangiectasia, associated with a high incidence of malignancy and a DNA repair defect, have been shown to have chromosomal breaks, translocations, and rearrangements at the locus of the T_γ, T_β, and T_α genes.

A major series of studies have been performed on the critical role played by the receptor for IL-2 in the growth and differentiation of normal and neoplastic T cells. The activation of T cells initially involves the interaction of the antigen-specific T-cell receptor with a foreign antigen. This interaction triggers the T cells to enter the activated state. The activated T cell turns on the expression of the gene encoding IL-2 and secretes large quantities of this lymphokine. However, IL-2 cannot induce T cells to proliferate and differentiate unless it binds to specific IL-2 receptors. These receptors are absent from resting T cells. However, we have demonstrated that a second event occurs when foreign antigen binds to the antigen receptor on resting T cells: the activation of the gene that codes the IL-2 receptor. Thus, both the growth factor IL-2 and its receptor are absent on resting T cells, but following antigen activation the genes for both proteins become expressed. A monoclonal antibody, anti-Tac, was prepared which reacts with the IL-2 binding site of both high and low affinity IL-2 receptors. The anti-Tac monoclonal antibody was utilized to characterize the IL-2 receptor on phytohemagglutin (PHA)-activated normal lymphocytes. Anti-Tac immunoprecipitations of ^{125}I surface-labeled PHA-activated normal lymphocytes led to the identification of a diffuse band of 55,000 daltons as the putative IL-2 receptor. This protein was also identified in the immunoprecipitations from cells precultured with ^{3}H-labeled D-glucosamine, thus demonstrating that it is a glycoprotein. Furthermore, when cells were biosynthetically labeled with ^{35}S-methionine and the immunoprecipitates analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gel, a similar band was identified. In addition, bands of 113,000 daltons and approximately 180,000 daltons were also identified. Since the 113,000 dalton and 180,000 dalton peptides were not labeled by either radioiodine or by radiolabeled glucosamine, they do not appear to be surface membrane

receptors. We are performing studies to determine whether one or both may be part of a theoretical receptor complex and, therefore, co-immunoprecipitate due to strong hydrophobic interactions with the 55,000 dalton peptide.

cDNAs encoding the human IL-2 receptor have been molecularly cloned. For these studies, the human receptor for IL-2 was purified by immunoaffinity chromatography using the anti-Tac monoclonal antibody. Oligonucleotides were prepared on the basis of the sequence of the 29 amino acids. Using this probe, cDNAs hybridizing with mRNAs for the IL-2 receptor were cloned and subsequently expressed in COS-1 cells. This cloned cDNA encoded a peptide that bound to IL-2 and that was recognized by the anti-Tac monoclonal antibody. The deduced amino acid sequence of the IL-2 receptor indicates that it is a peptide composed of 272 amino acids including a 21-amino-acid signal peptide. The receptor contains two potential N-linked glycosylation sites as well as multiple, possibly O-linked, carbohydrate sites. Furthermore, there is a single hydrophobic transmembrane region of 19 amino acids and a very short, 13-amino-acid cytoplasmic domain. The cytoplasmic domain of the IL-2 receptor appears to be too small for enzymatic function, thus the receptor differs from known growth factor receptors that are tyrosine kinases. Potential phosphate acceptors (serine and threonine, but not tyrosine) are present within the intracytoplasmic domain.

Utilizing the anti-Tac monoclonal antibody, we have defined those lymphocyte functions that require an interaction of IL-2 with its inducible receptor on activated T cells. As noted previously, anti-Tac inhibited T-cell proliferation induced by soluble, autologous, and allogeneic antigens. Anti-Tac inhibited generation of cytotoxic T lymphocytes on allogeneic cell cultures but did not inhibit their action once generated. Furthermore, anti-Tac inhibited the sequential development of late-appearing activation antigens on T cells. Finally, anti-Tac inhibited the production of immunoglobulins by B cells stimulated by polyclonal activators. Over the past year, we have extended these functional studies of anti-Tac to an analysis of the in vitro generation of suppressor T lymphocytes. We demonstrated that anti-Tac inhibits concanavalin A-induced suppressor T-cell generation; partially inhibits the generation of suppressor T cells in cultures of polymorphonuclear leukocytes cultured with the Epstein-Barr virus; and inhibits the generation of suppressor T cells induced by high antigen concentrations in a sheep red blood cell antigen-specific antibody assay system. In additional independent studies, we demonstrated that anti-Tac inhibits specific antibody synthesis in in vitro culture systems that do not involve polyclonal activators. Specifically, anti-Tac inhibited the anti-sheep red blood cell response, the response to a rickettsial antigen, and the response to haemophilus influenza antigen in such in vitro culture assays using human peripheral blood mononuclear cells.

A series of studies were initiated to determine the expression of IL-2 receptors identified by the anti-Tac monoclonal antibody in normal and malignant T cells. Normal resting T cells in the circulation, lymph nodes, and spleen, as well as circulating Sézary leukemic T cells and acute T-cell leukemic cells, did not express IL-2 receptors. In contrast, the leukemic T-cell populations of all patients with ATL examined expressed the Tac antigen. ATL is a malignancy of mature T cells that has a tendency to infiltrate the skin. This is a disease

with a very aggressive course that is often complicated by hypercalcemia and pulmonary infiltrates. It is the disorder that is caused by the human type C retrovirus HTLV-I. The IL-2 receptor expression on the ATL cells differs from that on normal T cells. First, unlike normal T cells, ATL cells do not require prior activation to express IL-2 receptors. Furthermore, using a ^{3}H-anti-Tac receptor assay, HTLV-I-infected leukemic T-cell lines characteristically expressed 5- to 10-fold more receptors per cell than did maximally PHA-stimulated T lymphoblasts. In addition, whereas normal stimulated human T lymphocytes maintained in culture with IL-2 demonstrated a rapid decline in receptor number, the ATL cells did not show a similar decline. Furthermore, we have shown that some, but not all, HTLV-I-infected continuous T-cell lines display aberrantly sized IL-2 receptors. It is conceivable that the constant presence of high numbers of IL-2 receptors on ATL cells and/or the aberrancy of these receptors may play a role in the pathogenesis of the uncontrolled growth of these malignant cells. As noted above, the T-cell leukemias caused by HTLV-I, as well as all T- and B-cell lines infected with HTLV-I, universally express large numbers of IL-2 receptors. A recent report by Haseltine and colleagues suggests a potential mechanism for this association between HTLV-I and IL-2 receptor expression. In addition to the gag, pol, env, and LTR (long terminal repeat) sequences common to other groups of retroviruses, HTLV-I contains an additional genomic region between env and the LTR referred to as pX. Haseltine and colleagues demonstrated this pX region encodes a 38,000 to 42,000 dalton protein termed LOR (long open reading frame) protein that may act as a transacting regulator of transcription. They demonstrated that the LOR protein acts on the LTRs (promoter and regulatory sequences) of HTLV-I, -II, and -III, stimulating transcription. We are examining the possibility that the LOR protein could theoretically also play a central role in increasing the transcription of host genes such as the IL-2 receptor gene involved in T-cell activation and HTLV-I-mediated leukemagenesis. With the cloning of the gene encoding the IL-2 receptor, this hypothesis that the LOR protein acts as a transacting regulator of transcription of the IL-2 receptor gene can now be tested.

We have initiated clinical trials to evaluate the efficacy of intravenously administered anti-Tac monoclonal antibody and Pseudomonas-conjugated anti-Tac antibody in the treatment of patients with ATL. The scientific basis for these studies is the observation that ATL cells express the Tac antigen, whereas normal resting T cells and their precursors do not. Three patients with ATL have been treated with intravenously administered unmodified anti-Tac. No patient suffered any untoward reactions nor did they produce antibodies reactive with mouse immunoglobulin or the idiotype of the anti-Tac monoclonal. Two patients with a very rapidly developing form of ATL had a very transient response; however, therapy of the other patient was followed by a 6-month remission as assessed by regression of skin lesions, by routine hematological tests, by immunofluorescence analysis, and by molecular genetic analysis of the arrangement of the T-cell receptor β chain genes. Six months following initial remission, the leukemia recurred with the reappearance of circulating leukemic cells identified by immunofluorescence and molecular genetic analysis and with the development of large malignant skin lesions. A second course of three infusions of 20 mg each of anti-Tac over a 10-day period was followed by the virtual disappearance of skin lesions and an over 90% reduction in the number of circulating leukemic

cells. Three months subsequently, leukemic cells were again demonstrable in the circulation. At this time the clonal population of cells manifested the same insertion position of the HTLV-I virus, indicating they were derived from the original clone of leukemic cells. However, they showed an arrangement of the T_{β} chain genes that differed from that observed previously with the leukemic cells of this patient. Thus, this leukemic clone had shown a progression of the arrangement of the T-cell receptor genes. At this time the leukemia was no longer responsive to infusions of anti-Tac and the patient required further chemotherapy.

Although there was no toxicity associated with the anti-Tac therapy, it is felt that in most cases it would be necessary to couple a toxin, isotope, or chemotherapeutic agent to anti-Tac. Thus, these studies have been extended in vitro by examining the ability of anti-Tac coupled to toxins or isotopes to kill cells of the T-cell line HUT 102-B2 derived from an ATL patient. The addition of anti-Tac antibody, coupled to toxin A chain of ricin, effectively inhibited protein synthesis of the HUT 102 line, but was much less effective at inhibiting protein synthesis by T-cell lines that did not bear the Tac antigen. Furthermore, the toxicity of the antibodies conjugated with ricin A was inhibited if unlabeled anti-Tac or IL-2 was added to the system. In parallel studies, anti-Tac has been successfully conjugated to the alpha-emitting radionuclide bismuth-212 by use of a bifunctional ligand, the i-butylcarboxycarbonic anhydride of diethylenetriaminepentaacetic acid. Specificity of the ^{212}Bi-labeled anti-Tac was demonstrated for the IL-2 receptor-positive adult T-cell line HUT 102-B2 by protein synthesis inhibition in clonagenic assays. Activity levels of 0.5 µCi, equivalent to 12 rads/ml of alpha irradiation targeted by anti-Tac, eliminated greater than 98% of the HUT 102-B2 proliferative capabilities with only modest effects on IL-2 receptor-negative cell lines. Specific cytotoxicity was blocked by excess unlabeled anti-Tac but not by 1 mg/ml human IgG. In addition, an irrelevant, isotope-identical, control monoclonal antibody labeled with ^{212}Bi was unable to target alpha irradiation to cell lines. Therefore, ^{212}Bi-anti-Tac is a relatively effective and specific immunocytotoxic reagent for the removal of IL-2 receptor-positive cells. These experiments thus provide the preliminary scientific basis for the use of alpha-emitting radioisotopes (radionuclides) in immunotherapy.

The most extensive therapeutic studies have been those performed in collaboration with Ira Pastan examining the efficacy of Pseudomonas exotoxin (PE) coupled to anti-Tac in selectively inhibiting the protein synthesis and the viability of Tac-positive ATL lines. PE is a bacterial toxin that kills cells by inhibiting protein synthesis. To accomplish this, PE must reach the cytoplasm where it catalytically inactivates elongation factor-2 and thereby inhibits protein synthesis. Human cells and other primate cells are naturally resistant to unmodified PE probably because it binds to them poorly. PE was coupled to antibodies in a reaction in which PE was treated with immunothiolane. The immunothiolane treatment alters the PE so its native cytotoxicity is markedly decreased, whereas its ability to inactivate elongation factor-2 in cell extracts is unimpaired. This creates an ideal reagent to couple to anti-Tac so that anti-Tac can carry PE to cells with Tac receptors. PE-anti-Tac was shown to enter these cells by the endocytic pathway and some of the conjugate escapes into the cytosol where

it inhibits protein synthesis and kills the cells. When Tac-positive cell lines from patients with ATL were cultured with 5×10^{-11} M PE-anti-Tac for 24 hours, protein synthesis was inhibited by 50%, and by 48 hours all these cells were dead. In contrast, at 5×10^{-9} M, PE-anti-Tac did not kill any Tac-negative cells in culture. Furthermore, only modest toxicity was observed when PE-anti-Tac was administered to cynomologus monkeys. Clinical trials with the administration of PE-anti-Tac to patients with ATL have been initiated. It is hoped that the Pseudomonas-conjugated anti-Tac antibody immunotherapy will prove effective in this, until now, universally fatal ATL.

As indicated above, human T cells activated with mitogens or antigens acquire a number of new or reexpressed surface antigens, including the IL-2, transferrin, and insulin receptors. We have now demonstrated that insulin-like growth factor (IGF) receptors type I and type II are also expressed on activated T cells. These studies were based on studies of specific binding of iodopeptides, relative potencies of unlabeled peptide in inhibiting iodopeptide binding, chemical crosslinking of these iodopeptides with their receptors, and immunoprecipitation of surface-labeled type I receptors by a monoclonal antibody. Analysis of crosslinked radiopeptide receptor complexes on activated T cells by SDS-polyacrylamide gel electrophoresis under reducing conditions demonstrated IGF-I crosslinked to a 135,000 dalton α subunit (type I receptor) and radiolabeled rIGF-II crosslinked to a 250,000 dalton (type II) and a 135,000 dalton (type I) band, of which only the 135,000 dalton band was inhibited by the addition of unlabeled insulin. Thus, human activated T cells were shown to express both type I and type II IGF receptors which may play an important role in T-cell growth, differentiation, and function.

A final area of major effort over the past few years has been directed toward defining the events of cellular differentiation and interaction involved in the specific, circulating immune response. These studies have placed emphasis on the defects of B-cell maturation, of helper T-cell function, and especially of suppressor T-cell and macrophage action in the pathogenesis of the immunodeficiency of patients who have an associated high incidence of malignancy. In one facet of these studies, we have been defining a lymphokine termed SISS-B, which inhibits B-cell proliferation without inhibiting T-cell proliferation or function. In previous studies, we used T cells activated by lectins, T-cell lines established from these lectin-stimulated T cells that were maintained in IL-2, as well as human T-T-cell hybridomas. Each of these approaches was satisfactory for the demonstration that a lymphokine of interest existed and for preliminary characterization of this soluble suppressor of immunoglobulin production. However, none of these lines or hybridomas was sufficiently stable to produce large quantities of the lymphokine for purification or for the production of cDNA libraries to molecularly clone and express the gene producing this lymphokine. We thus turned to T-cell lines immortalized by the virus HTLV-I. Using cocultures between normal T cells and HTLV-I-expressing T cells, an array of normal T-cell clones immortalized by HTLV-I that varied in terms of the lymphokine they produced have been obtained. We chose a line, CR-II-2, that produced the lymphokine of interest--one that inhibited immunoglobulin synthesis by pokeweed mitogen-stimulated B cells and inhibited B-cell proliferation stimulated by Staphylococcus aureus Cowan strain I organisms, but that did not inhibit T cells in terms

of their proliferation to PHA, nor in terms of their cytotoxic function. Furthermore, efforts are being made by groups collaborating with us to obtain cDNA clones of part of the HTLV-I virus, the pX region, that may be useful in obtaining immortalized T cells by transfection of the cDNAs into nonsecreting T-cell lines. The next major elements of this specific project, that is, the purification of the soluble suppressor that inhibits immunoglobulin synthesis and the cloning of cDNAs recognizing and encoding the CR-II-2 suppressor lymphokine have been initiated.

Significance to Biomedical Research and the Program of the Institute:

The studies of leukemias using recombinant DNA technology are providing insights into the earliest events in B- and T-cell maturation and are aiding in the classification of malignancies that were previously of controversial origin. They are providing methods for the early diagnosis and the definition of the lineage of lymphoid malignancies and are providing insights into the pathogenesis of the disorders of maturation of malignant B- and T-lymphoid cells. Taken in conjunction with studies of transposed oncogenes, these studies are providing insights into the nature of the malignant transformation itself. The studies related to the definition of the IL-2 receptor are important in our understanding of the mechanisms involved in the control of T-cell proliferation and in the differentiation of T cells into cytotoxic, suppressor, and helper T-cell populations. Furthermore, they are providing important new information concerning the pathogenesis of the uncontrolled growth of leukemic T cells of patients with HTLV-associated T-cell leukemia. They have provided the scientific basis for a new approach to the therapy of human malignancy: the use of monoclonal antibodies to a growth factor receptor expressed on malignant but not normal resting T cells. These studies are among the earliest to utilize toxin conjugates of a monoclonal antibody in the treatment of human malignancy. Finally, the development of techniques for the study of the effect of helper and suppressor T cells on the maturation of B cells and their application to the study of patients with immunodeficiency disorders has been of great value in defining the critical stages of B-cell maturation and especially on the nature of the network of immunoregulatory cells that controls this maturation process. A series of new immunodeficiency disorders that are associated with a high incidence of malignancy have been defined. The studies of suppressor lymphokines may be of importance not only in understanding the normal mechanisms of negative regulatory control, but also in providing biologically active molecules that could be used to inhibit unwanted immunoglobulin synthesis. In general, these studies are providing the scientific basis for the development of rational strategies for the therapy of immunodeficiency, autoimmune, and malignant disease.

Proposed Course of Research:

The analysis of the IL-2 receptor on normal and malignant T cells will be continued. Special emphasis will be made on studies of chains potentially associated with the 55,000 dalton IL-2 binding protein already identified. The distribution of IL-2 receptors on nonlymphoid cells will be analyzed following our preliminary observation that nonlymphoid cells may manifest IL-2 receptors. Furthermore, the role of IL-2 interaction with its receptor on T cells in the

development of a sequential cascade of receptors, including somatomedin and insulin receptors as well as transferrin receptors, Ia antigens, and very late antigens, will be extended. The role of IL-2 in antibody production and B-cell growth and differentiation will be examined now that we have demonstrated that activated B cells manifest a receptor for IL-2. The efficacy of anti-Tac monoclonal antibody and anti-Tac monoclonal coupled to toxins in the treatment of patients with Tac-positive ATL will be extended. Furthermore, a series of studies have been initiated to study the effect of anti-Tac in organ transplantation protocols. These studies are based on the observation that anti-Tac inhibits the mixed leukocyte reaction and the generation of cytotoxic T cells and that Tac-positive cells may be present in transplanted organs undergoing rejection. Furthermore, the efficacy of immunotherapy with anti-Tac unmodified and coupled to Pseudomonas toxin will be studied in patients with Tac-positive T cells involved in autoimmune disorders. Specifically, studies have been initiated on the effect of anti-Tac in the therapy of patients with aplastic anemia who have Tac-positive cells that inhibit marrow hematopoiesis. In additional studies special emphasis will be placed on the analysis of the immunoglobulin and antigen-specific T-cell receptor genes and their rearrangement. We will use analysis of the rearrangement of these genes to identify the lineage and clonality of neoplasms. Furthermore, we will extend these studies to aid in the diagnosis of neoplasia in patients that have abnormal infiltrates of the skin and those with disordered lymph node architecture. Furthermore, studies of patients with heavy chain disease will be extended to define these disorders at a genetic level. Analysis of autosomal recessive immunodeficiency diseases, as well as X-linked immunodeficiency diseases, using restriction fragment length polymorphism will be pursued to define these genetic immunoglobulin deficiencies in terms of the chromosomal site of the abnormal gene. Further studies will be performed on patients with immunodeficiency diseases using probes for genes that are uniquely expressed in T or B cells but not the opposing cell. Such clones have been obtained using subtraction library approaches. Finally, we will extend the efforts to purify, determine the amino acid sequence, and molecularly clone the gene for the lymphokine that suppresses immunoglobulin synthesis without altering T-cell proliferation.

Honors and Awards:

Peter Horvath Memorial Lecture - Atlantic Dermatology Society
Herman Beerman Lecture - Society for Investigative Dermatology
Jessie Horton Koessler Lecture - The Institute of Medicine of Chicago
Election to National Academy of Sciences

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04004-23 MET

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulatory Functions of Amino Acids on Ribonucleotides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	James M. Phang	Section Head	MET,NCI
	G. Alexander Fleming	Medical Staff Fellow	MET,NCI
	Marsha Merrill	Guest Researcher	MET,NCI

COOPERATING UNITS *(if any)*

Grace C. Yeh, CPB, DCT, NCI
David Valle, M.D., Johns Hopkins Hospital School of Medicine, Baltimore, MD

LAB/BRANCH

Metabolism Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	3	3

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The metabolism of proline and pyrroline-5-carboxylate (P5C) provides a mechanism for the intercompartmental, intercellular, and interorgan transfer of redox potential. Mediated by the transfer of redox potential, P5C stimulates the pentose phosphate pathway, phosphoribosyl pyrophosphate (PRPP) synthesis, and nucleotide production. This mechanism links amino acid and nucleotide metabolism. This effect of P5C has been shown to be synergistic to the effect of growth factors on ribonucleotide synthesis, and suggests that P5C may mediate hormonal effects and, indeed, may act as a "primitive hormone." Such a role for P5C as an intercellular communicator has been supported by studies in normal human volunteers. P5C is found in human venous plasma (0.40 ± 0.12 μM). More importantly, the levels are responsive to nutritional perturbation increasing 10-fold to 3-5 μM following a protein meal. Thus, P5C may serve as a mechanism to link nutritional factors to growth factor action. Finally, P5C has been used as a probe to uncover redox abnormalities in tumor cells resistant to adriamycin. The transfer of oxidizing potential into the pentose phosphate pathway mediated by P5C is 4- to 5-fold greater in adriamycin-resistant cells than in wild type cells.

Project Description

Objectives:

An understanding of metabolic regulation has greatly contributed to recent advances in elucidating the mechanisms of malignant transformation. Although studies of metabolic regulation have emphasized the function of macromolecules, recent work has emphasized several small molecules, e.g., fructose-2, 6-bisphosphate, diacylglycerol, and inositol phosphate, as mediators of important regulatory mechanisms. In fact, these may be new members in the ever-growing family of small molecules which mediate metabolic signals. Our project emphasizes amino acid metabolites as mediators of regulatory events. Proline and its metabolic intermediates have been of special interest because they have unusual, even unique features. Functioning as a redox couple, proline and P5C can regulate redox-dependent metabolic pathways and markedly stimulate the production of PRPP and nucleic acids. We hope to link this mechanism to events occurring at the cell membrane and to show that this mechanism may be involved in some of the pleiotypic metabolic derangements found in tumors and transformed cells.

Methods Employed:

A wide spectrum of methods are used to elucidate the molecular mechanisms by which P5C acts as a regulatory molecule. These include the quantitation of metabolites, e.g., PRPP, nucleotides, P5C, and precursors and products of P5C. High pressure liquid chromatography is used to quantitate specific nucleotides and to study the incorporation of radiolabeled precursors into nucleotides. The enzymes of P5C synthesis and degradation are assayed with an enzyme-coupled assay for measuring small quantities of P5C. The assay is sensitive to the picomolar range and has been useful in measuring P5C concentrations in biologic fluids. To elucidate the enzyme mechanisms mediating the effects of P5C, we are purifying P5C reductase using affinity chromatography and are using polyacrylamide gel electrophoresis to characterize the molecular basis for kinetic differences in enzyme from different cell sources as well as in cells undergoing mitogenic activation.

Major Findings:

Clinical and basic research goals: Our laboratory has shown that proline and its metabolite, P5C, regulate major metabolic pathways. P5C, the obligate intermediate in the direct interconversions of proline, ornithine, and glutamate, can initiate a redox-dependent metabolic cascade. This cascade includes: 1) the activation of the pentose phosphate pathway, 2) increased formation of PRPP, and 3) increased production of nucleotides. Furthermore, we have shown that the interconversions of proline and P5C constitute a metabolic cycle in which oxidizing potential in the form of P5C can be generated and transferred between cellular compartments as well as between different cells with asymmetric enzyme capacities. Thus, the proline-P5C cycle can catalytically generate oxidizing potential and open metabolic gates necessary for cellular activation. Based on our findings, we hypothesized that proline and P5C may act as "transabolic regulators" for intercompartmental and intercellular communication. The term

"transabolic" denotes that proline and P5C are metabolized in the process, but the target of the regulation is a pathway distant and distinct from the metabolism of proline or P5C. We propose that derangements of this regulatory mechanism may be involved not only in malignant transformation but also in the pathogenesis of certain inborn errors of metabolism. For example, defective proline-P5C redox transfer mechanisms may be involved in the neurological disorder found in some patients with type II hyperprolinemia and in the ocular pathology associated with gyrate atrophy of the choroid and retina.

We previously showed that the synthesis of PRPP and ribonucleotides is stimulated by P5C. This intermediate in the interconversions of proline, ornithine, and glutamate can activate the pentose phosphate pathway by a redox-dependent mechanism, thereby serving as a metabolic interlink between amino acids and ribonucleotides. We have focused on the physiologic effects of this newly discovered regulatory mechanism. First, we showed an interaction between the P5C-mediated mechanism and the effect of growth factors on ribonucleotide synthesis. Second, we identified P5C as a circulating molecule in human plasma and showed that the levels of this intercellular communicator are sensitive to dietary perturbation.

There are two response patterns in the interaction between P5C and growth factors. The response to a progression factor, multiplication stimulating activity (MSA)--a rat IGF-II--differed from that to a competence factor, platelet-derived growth factor (PDGF). Using purified MSA, the effect of P5C was additive to that of MSA on PRPP levels. In marked contrast, the effect of P5C increased synergistically as a function of PDGF concentration. At saturating concentrations of PDGF, the addition of P5C markedly increased the levels of PRPP over that of PDGF alone. Treatment with PDGF increased the P5C effect 8- to 10-fold over its effect in quiescent cells. Even at concentrations of PDGF which are inadequate to stimulate PRPP, the effect of P5C is markedly augmented. These patterns of response suggest that P5C and PDGF stimulate PRPP by different mechanisms but that P5C and MSA may work by similar mechanisms.

If the P5C-mediated mechanism contributes to the MSA effect on PRPP synthesis, one would expect a deficient response to MSA in a cell line unable to synthesize P5C. Using a clone of Chinese hamster ovary cells which lack the enzymes of P5C synthesis, we found that this indeed was the case. The effect of MSA on PRPP was blunted in this cell line. More importantly, the response was restored to normal with added P5C. Thus, endogenously synthesized P5C may mediate, at least in part, the stimulation of PRPP synthesis by MSA.

Since the synergistic stimulation of PRPP by PDGF and P5C suggested that they work through different mechanisms, we examined whether they might differ in their sensitivity to cycloheximide, an inhibitor of protein synthesis. Cells were incubated wih 0.5 mM cycloheximide for 30 min before the addition of P5C or PDGF. Under these conditions, protein synthesis was inhibited by 85%. We found that cycloheximide had no effect on either control or P5C-stimulated levels of PRPP in the absence of growth factors. The effect of PDGF, however, was markedly decreased by cycloheximide. The augmentation of the P5C effect by PDGF, interestingly, was not inhibited by cycloheximide. Thus, although the

stimulatory effect of PRPP by PDGF requires either a newly synthesized or a short half-lived protein, the PDGF-mediated augmentation of the P5C effect on PRPP does not.

The in vitro interaction between growth factors and added P5C would be physiologically relevant if P5C is a circulating intercellular communicator. Indeed, this is the case. In normal human volunteers, the fasting level of P5C in venous plasma is 0.40 ± 0.12 μM. Of special interest, the level can increase 10- to 15-fold with dietary perturbation. Frequent blood samples were obtained for P5C levels following a controlled isocaloric intake of carbohydrates, protein, and fats. Accompanying a meal high in protein, the levels of P5C can rise to 3-5 μM, concentrations which can have marked effects on PRPP levels in intact cells in vitro. This finding, together with the observed in vitro synergism between P5C and growth factors, may provide an approach for understanding the nutritional effects of high protein intake on tumor initiation and promotion.

Significance to Biomedical Research and the Program of the Institute:

The linkage of the proline-P5C redox couple to glucose and nucleotide metabolism has a number of implications. This linkage is the only known mechanism by which naturally occurring metabolic intermediates can activate the pentose phosphate pathway, PRPP formation, and ribonucleotide synthesis. The recently discovered synergism between P5C and growth factors in stimulating PRPP synthesis suggests that the proline-P5C system may be involved in the mechanism by which growth factors activate ribonucleotide synthesis. This finding is especially significant in oncogenesis because the growth factor axis has been implicated. Similarly, markedly augmented ribonucleotide synthesis has been recognized as a metabolic feature of tumors.

The redox effects of the proline-P5C system also provide a conceptual framework for studies in type II hyperprolinemia and gyrate atrophy. In the former, the neurological abnormalities may be related to redox derangements due to high levels of circulating P5C. In the latter, the inability to form P5C from ornithine, especially in ocular tissues, may result in a deficiency of ribonucleotide and pyridine nucleotide and in the lenticular and retinal pathology prominent in this disease.

Proposed Course of Research:

Recently identified mechanisms of oncogenesis have reemphasized the importance of metabolic regulation and intermediary metabolism. Our studies have introduced the proline-P5C system as "transabolic" regulators of the redox state and ribonucleotide synthesis. During the coming year we will focus on the following areas:

1) Molecular mechanisms of the P5C-mediated interaction with growth factors: The finding that PDGF and P5C synergistically stimulate PRPP synthesis makes this interaction an attractive model, especially since a number of the known effects of PDGF are mediated by the activation of protein kinase C by diacyl-

glycerol. In preliminary studies we found that phorbol myristate acetate (PMA) is a potent stimulator of PRPP synthesis. Since PMA acts by binding to and activating protein kinase C, it is likely that the stimulation of PRPP by PDGF is working through this axis. Continuing our studies in intact cells we will try to pinpoint the step(s) at which P5C interacts with PDGF by using PMA, diacylglycerol, and inhibitors of ion fluxes, e.g., amiloride and ouabain.

More direct studies are required to define the mechanism of the PDGF-P5C synergism. Studies on the interaction of P5C or P5C reductase with components of the PDGF cascade may be a useful approach. This can be done using cell-free systems. Direct effects of P5C on PDGF receptors, phospholipase C, and protein kinase C are interesting possibilities. It is also possible that cellular localization or protein-protein interaction involving P5C reductase may mediate the PDGF-P5C synergism.

Although crude cellular components can be used for many of these studies, purified proteins would be a more direct approach. The availability of both P5C reductase protein and specific antibodies would be immensely helpful. We have made considerable progress in the purification of P5C reductase from human erythrocytes. Using DEAE cellulose followed by affinity chromatography, we have purified the enzyme 15,000-fold from human red cells. The obstacle confronting us is that the enzyme protein is in very low abundance. Large quantities (perhaps 50 liters) of blood are necessary to obtain 5-10 μg of purified protein. We think that this goal can be reached in spite of this logistical obstacle.

2) P5C as an intercellular communicator: The hypothesis that P5C can function as an intercellular communicator has been supported by the finding that P5C is a nutritionally responsive molecule which circulates in human plasma. We will first characterize the nutritional and physiologic factors which influence P5C levels. We will then study patients with disease to identify abnormal populations.

3) The pathophysiologic role of P5C in human diseases:

a) Type II hyperprolinemia. Measurements of P5C levels in plasma have shown that they are markedly elevated in this metabolic disorder. Furthermore, we have shown that the arteriovenous oxygen gradient in peripheral tissues is markedly reduced. We are using cultured fibroblasts from patients to study the mechanism of this abnormal energy metabolism and to ascertain whether the abnormality is related to the inherited deficiency in P5C metabolism.

b) Gyrate atrophy. The finding of high concentration of P5C in rabbit aqueous humor suggests that P5C may play a special role in ocular tissues. The inability to synthesize P5C from ornithine may result in the retinal degeneration and lenticular opacities found in this disease. Using the rabbit lens, we will quantitate the incorporation of radiolabeled hypoxanthine and nicotinic acid into purine nucleotides and pyridine nucleotides, respectively. Specific nucleotide products will be recovered and quantitated on high pressure liquid chromatography. The effect of P5C on these formation rates may reveal that the

transabolic effect of P5C on nucleotides is especially important for ocular tissues.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04015-14 MET

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development and Function of Humoral and Cellular Immune Mechanisms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. Michael Blaese	Section Head	MET,NCI
	Andrew V. Muchmore	Senior Investigator	MET,NCI
	Alfred D. Steinberg	Senior Investigator	A&R,NIAMDD
	Steven E. Straus	Senior Investigator	LCI,NIAID
	Fred Wang	Medical Staff Fellow	MET,NCI
	Akihiro Yachie	Visiting Fellow	MET,NCI
	Basil Golding	Medical Staff Fellow	MET,NCI

COOPERATING UNITS *(if any)*
LCI,NIAID
A&R,NIAMDD

LAB/BRANCH
Metabolism Branch

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7	5	2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The Cellular Immunology Section has continued and extended its studies of the immunobiology of herpes group viruses. Our long-term investigations of the Epstein-Barr virus (EBV) have recently focused on the relationship of this virus with chronic diseases such as rheumatoid arthritis and chronic infectious mononucleosis. EBV is a unique viral pathogen for man in that its target cell for infection is the immune system itself. Not only are B lymphocytes infected by the virus, but once infected they become functionally activated to proliferate and secrete immunoglobulin (Ig) and significantly, they acquire the property of autonomous growth as well. In addition, like other herpes viruses, EBV persists in B cells for life after primary infection and retains its capacity to activate these cells at any time. These properties present a considerably more complex problem of host defense than encountered with other pathogens, and the body has evolved a broad array of humoral and cellular mechanisms to control this pathogen and regulate its effects on its lymphoid cell target. This research project has identified both suppressor and cytotoxic mechanisms of control which evolve from EBV-nonspecific mechanisms in acute primary infection to EBV antigen-specific mechanisms which appear during convalescence. In patients with rheumatoid arthritis, EBV-specific suppressor T-cell function is deficient, while in patients with chronic mononucleosis, excessive suppressor activity persists abnormally. Cytomegalovirus (CMV) is another herpes group virus which stimulates striking Ig production *in vitro*. In contrast to EBV, however, CMV is a T-cell-dependent polyclonal B-cell activator and stimulates Ig production only in the presence of CMV-immune T cells. In patients with congenital CMV infection, polyclonal hypergammaglobulinemia appears to be the result of chronic stimulation of this virus.

Project Description

Objectives:

The objectives of this study were to determine mechanisms important to the development and function of humoral and cellular immune responses in man and animals; to determine the nature of the deficiency in immune function characterizing such disease states as hypogammaglobulinemia, ataxia telangiectasia, the Wiskott-Aldrich syndrome, intestinal lymphangiectasia, and advanced malignancy; and to develop approaches to prophylaxis and/or therapy of the infections and neoplastic disease frequently associated with defective immune processes. This plan is directly related to Objective 2, Approach 1 of the National Cancer Plan.

Methods Employed:

Antibody responses of experimental animals and man were determined after immunization with a variety of antigens and the antibodies were detected by standard techniques or methods developed in our laboratory. Cellular immune responses were measured by delayed hypersensitivity skin testing. Proliferative responses in vitro were tested using nonspecific mitogens, specific antigens, and allogeneic cells in mixed lymphocyte culture. Ig secretion by activated lymphocytes was measured by a reverse hemolytic plaque assay and this test was applied to cultured cells stimulated with a variety of mitogens, viruses, and chemical agents. Studies of the characteristics of various cell surface receptors on immunocompetent lymphocytes and monocytes-macrophages utilized autoradiography, immunofluorescence microscopy, cellular rosette formation, and immunohistochemistry.

Major Findings:

To determine the frequency of endogenously EBV-infected B cells capable of transforming into continuously growing cell lines, and to further characterize the mechanisms which control these cells, we have developed sensitive techniques for determining the rate of spontaneous B cell transformation in the human peripheral blood lymphocyte population. Using limiting dilution cultures of purified B cells grown on a feeder layer of autologous irradiated T cells, the frequency of endogenously EBV-infected B cells in seropositive normal subjects capable of spontaneous transformation was found to range from 1 to 10 cells per million B cells. In EBV seronegative adults and children, as expected, no spontaneously transforming B cells were found. In patients with acute EBV infection (i.e., infectious mononucleosis) 500 to 5000 cells per million B cells spontaneously transformed. Patients with profound T-cell deficiency based on congenital or acquired immunodeficiency diseases or as a consequence of immunosuppressive therapy have developed EBV-induced polyclonal B-cell malignancies. Using this powerful technique for the quantitation of endogenously EBV-infected B cells, we are presently studying patients treated with intensive combination cancer chemotherapy as well as patients treated with cyclosporin A as a single agent, to determine the effects of these drugs on the in vivo control mechanisms for EBV. In eight patients receiving chronic cyclosporin A therapy for inflammatory uveitis, the frequency of endogenously EBV-infected B lymphocytes was similar

to that found in untreated EBV seropositive normal subjects. These patients were receiving therapy with cyclosporin A at dosages comparable to that used in cardiac allograft recipients who have developed a strikingly high frequency of B-cell neoplasia thought to be associated with EBV. We will have to continue our studies to observe the effects of prolonged cyclosporin A treatment, since these preliminary results are somewhat unexpected and suggest that additional factors not present in cyclosporin A treated patients who are not receiving organ allograft may be important to development of poorly controlled B-cell proliferation and activation in patients treated with this potent drug.

In addition to its association with certain B-cell lymphomas and acute infectious mononucleosis, EBV also has an as yet undefined association with the common form of the adult rheumatoid arthritis. For example, patients with rheumatoid arthritis have high titers of antibody to EBV-associated antigens. When cultures of peripheral blood lymphocytes from normal EBV seropositive donors are infected with EBV, initially the virus activates B cells to produce Ig which peaks at 8 to 10 days of culture and then this production rapidly falls as a consequence of the EBV-immune suppressor T cells present in these donors. By day 14 of culture, the presence of EBV-immune suppressor T cells inhibits Ig production in these cultures by 90% compared with EBV-infected B cells cultured alone. In striking contrast, when lymphocytes from EBV seropositive rheumatoid arthritis patients are stimulated with EBV in vitro, Ig production progressively increases throughout the 14-day culture period indicating a defect in EBV-specific suppressor T-cell activity in these patients. When the number of endogenously EBV-infected cells in these rheumatoid arthritis patients was determined by precursor frequency analysis, they were found to have an average of 10 times as many spontaneously transforming B cells as the normal subjects. Thus, the in vitro demonstration of defective suppressor T-cell activity in these patients correlates well with the presence of an increased burden of EBV-infected B cells in vivo and again suggests that many of the immune abnormalities in patients with rheumatoid arthritis are related to defective T-cell control of this virus and the consequent effects of virus-induced B-cell activation.

To further address the relationship between B-cell activation and EBV infection, limiting dilution analysis was used to measure the frequency of precursors for EBV-induced Ig production compared with EBV-induced B-cell transformation. In these experiments the vast majority of cells which were induced to transform also produced Ig. However, many cultures produced Ig without concomitant transformation. In fact, twice as many B cells secreted Ig in response to virus stimulation as were transformed. Kinetic analysis showed that the nontransformed B-cell clones secreted Ig for 4 to 6 weeks and then ceased, while transformed B cells produced Ig throughout the 12 weeks of observation. A strong correlation between isotype commitment and transformation was also found. The rate of transformation among B cells committed to IgM production averaged 65%, while transformation of those B-cell clones producing IgA and IgG averaged only 20%.

Earlier we showed that T cells from EBV-immune donors have a characteristic type of "late suppression" following exposure to infected B cells in vitro. EBV-

stimulated B lymphocyte cultures produce an exponentially increasing number of Ig-secreting cells during the first 3 weeks of culture. In the presence of immune T cells, such EBV-stimulated B cells show an identical response during the first 7 to 10 days of culture, followed by a striking decline in Ig production so that by 14 days cultures containing immune T cells are producing less than 10% of the Ig produced by cultures containing B cells alone. This "late suppression" is inhibited by monoclonal antibodies directed at the T cell antigen receptor (αT_3) and also by antibodies to the T-cell growth factor receptor (αTAC) on T cells. Depletion of T cells bearing the T_4 membrane antigen has little effect, while depletion of T_8-bearing cells removes this suppressor activity. Interestingly, removal of monocytes from this system increases the suppression observed by several fold so that monocytes appear to have a contrasuppressive effect on these T_8-bearing suppressor cells. However, addition of monocytes to T_8 cells did not reverse suppression unless T_4 cells were also present in the culture. Thus, the EBV-immune "late suppressor" phenomenon is an exceptionally complex interaction of T_8-bearing suppressor T cells which directly regulate Ig production of the B cells and the T_8 cells themselves are regulated by a contrasuppressor circuit consisting of monocytes and T_4-bearing T cells.

Another major effort in the laboratory has involved the study of a group of patients with a disorder that we have called chronic infectious mononucleosis. This disorder is characterized by a symptom complex dominated by overwhelming and disabling fatigue and malaise, with low grade fever, recurrent painful lymphadenopathy, prominent allergic symptomatology, and other constitutional complaints. About 50% of these patients date the onset of their disorder to an attack of acute EBV-induced infectious mononucleosis and the mean duration of illness in the group was 7.5 years. A relationship between this illness and EBV is suggested by a pattern of antibodies in these patients characteristic of active EBV infection, even though they had experienced their acute EBV infection years earlier. Thus, high titers of antibodies to VCA and EA are found, while antibodies to EBNA are absent or low in titer. Extensive clinical and immunologic evaluation of these patients has found remarkably few abnormalities. One striking finding is the presence of significantly depressed levels of one or more serum Ig in almost one-half of the group. Another finding has been the demonstration of supressor T cells capable of inhibiting pokeweed mitogen (PWM)-induced Ig production by allogeneic but not autologous mononuclear cells *in vitro*. This later finding was found in our initial group of 26 patients, but has not been a prominent feature of 18 additional subjects with a more homogeneous disease pattern currently undergoing a double-blind therapy trial with acylovir.

The Cellular Immunology Section has had a long-term interest in the effects of herpes group viruses on immune responses in man. In addition to our extensive studies of the effects of the EBV on human B-cell differentiation and the immunoregulatory T-cell mechanisms which control this virus, we have recently evaluated immunoregulatory mechanisms responding to infection with CMV. CMV is an opportunistic herpes virus which infects the majority of people by adulthood, but which is associated with disease primarily in the neonate and in immunocompromised hosts. It presents a major problem in patients receiving solid

organ and bone marrow transplantation and in patients with AIDS. We found that peripheral blood lymphocytes in culture responded to stimulation with CMV with impressive T-cell proliferation accompanied by B-cell activation to Ig synthesis. Ig production induced by CMV is polyclonal, includes all isotypes, and often equals the response induced by PWM or EBV. In contrast to EBV, however, CMV induced Ig production only in cultures containing T lymphocytes and further, only in donors who were immune to CMV. Ultraviolet-irradiated or heat-killed CMV are as efficient as infectious virus in inducing these in vitro responses, again in contrast to EBV where only viable infectious virus is stimulatory. Therefore, CMV as with another herpes group virus, EBV, is profoundly stimulatory for Ig production by human lymphocytes. However, while EBV stimulates B cells directly and induces Ig production in cells from immune or nonimmune donors, CMV is dependent on the presence of immune T cells for its ability to induce Ig production. This polyclonal T-cell-dependent B-cell activation induced by CMV may in part explain the hypergammaglobulinemia found in neonates with congenital CMV infection and adults with AIDS.

Significance to Biomedical Research and the Program of the Institute:

The present studies extend our understanding of the diverse, yet interrelated mechanisms contributing to the development and normal functioning of immune systems in animals and man. They indicate that the normal expression of immune function is dependent on multiple processes and that defects in immunity may be the result of factors influencing a variety of these processes. For example, dysfunction of the immune system can occur through such diverse factors as defects in differentiation on the one hand or through the mediation of exogenous viral pathogens on the other. Thus, human disease may result from a deficiency of immune elements as in certain forms of agammaglobulinemia or from an excess of certain immune functions as in other types of agammaglobulinemia associated with excessive suppressive T-cell activity. Suppressor T cell function may be part of a normal defense mechanism in such diseases as infectious mononucleosis, and a deficiency of normal suppressor T-cell activity may lead to the expression of certain autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus. Our new insights into the role of sugar-lectin interactions as immunoregulatory signals offer potential for precise definition of this communication process and for future therapeutic manipulation of these signals.

Proposed Course of Research:

Continue the studies directed toward the understanding of the pathways involved in the development and function of normal immune responses and the application of insights gained through such studies for the development of new approaches for the prevention and therapy of human disease.

Publications:

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Straus, S.E., Reinhold, W., Smith, H.A., Ruyechan, W.T., Henderson, D.K., Blaese, R.M., and Hay, J.: Endonuclease analysis of viral DNAs from varicella and subsequent zoster infections in the same patient. N. Engl. J. Med. 311: 1362-1364, 1984.

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nancy urine: A new immunoregulatory saccharide. J. Exp. Med. 160: 1672-1685, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04016-12 MET

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanism of Action of Insulin-Like Growth Factors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	S. Peter Nissley	Senior Investigator	MET,NCI
	Lynne Gaynes	Medical Staff Fellow	MET,NCI
	Joyce Haskell	Guest Researcher	MET,NCI
	Matthew M. Rechler	Senior Investigator	NIADDK
	Wayne Anderson	Senior Investigator	LTIB,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.75	3	2.75

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors B

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have attempted to develop monoclonal antibodies against the rat type II (insulin-like growth factor) IGF receptor. Assays that were devised to measure blocking and immunoprecipitating antibodies have been utilized to screen mouse sera and hybridoma supernatants. Mice immunized with cells or membranes containing the rat type II receptor have developed positive titers in their sera. However, when spleen cells from these mice have been fused with plasmacytoma cells and the resulting hybridomas screened for the production of receptor antibodies, the incidence of positive responses has been low and the few positive hybridoma supernatants have been weak, either of low titer or low affinity. We are currently using in vitro immunization techniques with the rat type II receptor and switching from rat to human type II receptor as a source of antigen for conventional hybridoma technology. While we previously were unable to demonstrate IGF-dependent phosphorylation of the type II receptor in whole cell labeling experiments with rat embryo fibroblasts, we have been able to demonstrate IGF-dependent phosphorylation in a rat liver cell line (BRL-3A2). One difference between the rat embryo fibroblasts and the rat liver cell line is that rat embryo fibroblasts produce IGF-II, raising the possibility that endogenously produced IGF-II causes phosphorylation of the type II receptor. Earlier we had reported that fetal rat fibroblasts produced IGF-II, but not IGF-I, whereas postnatal fibroblasts produced predominantly IGF-I. We have examined IGF production by human fetal and postnatal fibroblasts. In contrast to rat fibroblasts, the levels of IGF are much lower and there are no dramatic differences between the relative amounts of IGF-I and IGF-II in fetal versus postnatal fibroblasts. Chemical crosslinking experiments demonstrate that the human fibroblasts produce binding proteins analogous to subunits of both the small and large IGF binding proteins in human serum.

Project Description

Objectives:

The understanding of normal growth may be a prerequisite to understanding malignant growth. Indeed, the demonstration that platelet-derived growth factor (PDGF) and part of the epidermal growth factor (EGF) receptor are normal cellular homologues of transforming proteins of simian sarcoma virus (p28*sis*) and avian erythroblastosis virus (gp65*erbB*) respectively, provides a direct link between growth factors and the process of malignant transformation. The insulin-like growth factors (IGF)-I and -II are structurally related to insulin and are growth factors for a variety of cells in culture. IGF-I (somatomedin C) is the mediator of the anabolic effects of pituitary growth hormone (GH) and IGF-II may be a fetal growth factor in the rat; therefore, elucidation of the mechanism of action of IGFs *in vitro* should reflect the normal growth process *in vivo*. The objectives of the project are to purify one of the IGF, to study the regulation of production of IGF, to characterize cell surface receptors for IGF, and to understand postreceptor biochemical events. In addition, using human skin fibroblast we hope to identify patients who have end-organ resistance to IGF and identify the abnormality in the pathway.

Methods Employed:

MSA or multiplication stimulating activity (rat IGF-II) is being purified from serum-free medium conditioned by a rat liver cell line (BRL-3A) using ion exchange chromatography, gel filtration, and high pressure liquid chromatography (HPLC). Human fetal fibroblasts derived from skin and lung are being used to study the production of IGFs and IGF binding proteins. The IGFs produced by the human fetal fibroblasts are being characterized by bioassay (tritiated thymidine incorporation into DNA in chick embryo fibroblasts), radioreceptor assay, and radioimmunoassay. An IGF-II receptor is being purified from Swarm rat chondrosarcoma cells by subcellular fractionation, detergent solubilization from a membrane fraction, affinity chromatography on an IGF-Sepharose column, and Sepharose 6B gel filtration. Receptor purification is followed by measurement of binding radiolabeled IGF and separation of bound tracer from free tracer with albumin-coated charcoal. Phosphorylation of the type II IGF receptor is being studied by whole cell labeling with [^{32}P] orthophosphoric acid, purification of glycoproteins by wheat germ lectin affinity chromatography, and analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Phosphorylation of membrane fractions and solubilized receptor preparations were examined by using [γ-^{32}P] ATP and analysis on SDS-PAGE. Fibroblasts from patients with possible end-organ resistance to IGF are being cultured from punch biopsies. IGF receptors on these patients' fibroblasts are being characterized by competitive binding studies and biologic response to IGF is being measured (tritiated thymidine incorporation into DNA, amino acid and glucose transport).

Major Findings:

Phosphorylation of the IGF-II (type II) receptor: There are two types of IGF receptors. One type preferentially binds IGF-I compared to IGF-II and interacts

weakly with insulin. Affinity crosslinking studies have shown that the binding subunit of this type I IGF receptor has a Mr of 130,000 daltons, similar to the binding subunit of the insulin receptor. A second type of IGF receptor preferentially binds IGF-II compared to IGF-I and does not interact with insulin. Affinity crosslinking studies have shown that the binding subunit of this receptor has a Mr of 260,000 daltons. The EGF, insulin, and type I IGF receptors are phosphorylated in a ligand-dependent fashion and tyrosine kinase activity is an intrinsic property of the receptor. In contrast to these receptors, the type II IGF receptor is not autophosphorylated when incubated with [γ-^{32}P] ATP, nor does highly purified type II receptor exhibit tyrosine kinase activity toward exogenous tyrosine-containing substrate, poly (glu, tyr) 4:1. We asked whether the type II receptor was phosphorylated in intact cells.

We labeled the ATP pool of cells in culture (rat embryo fibroblasts, H-35 rat hepatoma, BRL-3A rat liver) with [^{32}P] orthophosphoric acid and then incubated the cells with IGF-II. The cells were solubilized with detergent and glycoproteins purified on a wheat germ lectin affinity column. The phosphorylated glycoproteins were analyzed by SDS-PAGE and fluorography. Among the phosphorylated bands examined under reducing and nonreducing conditions were species having the size of the type II receptor (Mr 250,000 daltons with reduction; Mr 220,000 daltons without reduction). Importantly, this phosphorylated species could be bound and eluted from an IGF-II affinity column. Whereas phosphorylation of the type II receptor was not dependent upon IGF in rat embryo fibroblasts, we were able to demonstrate an increase in phosphorylation of the type II receptor upon addition of IGF-II to BRL-3A2 rat liver cells. One potentially important difference between rat embryo fibroblasts and BRL-3A2 cells is that rat embryo fibroblasts produce substantial quantities of IGF-II; endogenously produced IGF-I could stimulate phosphorylation of the type II receptor. The role of phosphorylation in receptor function and/or regulation remains to be defined.

Development of monoclonal antibodies against the type II IGF receptor: A central question about the mechanism of action of the IGFs concerns which type IGF receptor is important in mediating a particular biologic response. Since both receptor types are present on most cells, antibodies directed against the IGF binding sites (blocking antibodes) would be useful in making this distinction. In addition, receptor antibodies could enable the purification of type II receptor on a large scale by affinity chromatography as well as being useful in biosynthetic studies.

Monoclonal antibodies are being developed using hybridoma technology. Mice are being immunized with intact Swarm rat chondrosarcoma cells, 100,000 x g membrane preparation, and purified receptor. Spleen cells are fused with mouse plasmacytoma cells (NS-1) and hybridomas selected in HAT medium. We have developed two assays to screen for receptor antibodies in mouse serum and hybridoma supernatants. The first assay identifies blocking antibodies by measuring the ability of serum or hybridoma supernatant to inhibit the binding of radiolabeled IGF-II to Swarm rat chondrosarcoma cells. The second assay detects antibodies directed against other parts of the receptor. Mouse serum or hybridoma supernatants are incubated with preformed radiolabeled IGF-II-solubilized receptor complex and immunoprecipitated with goat antimouse immunoglobulin.

However, when spleen cells from these mice have been fused with plasmacytoma cells and the resulting hybridomas screened for the production of receptor antibodies, the incidence of positive responses has been low and the positive hybridoma supernatants have been weak, either of low titer or low affinity. In addition, these positive hybridomas have not continued to be positive through the stages of cloning and production of ascites fluid. We are currently performing in vitro immunization with highly purified rat type II receptor to increase the likelihood of developing monoclonal antibodies. In addition, we are beginning to use preparations of human type II receptor for immunization since it is possible that the rat type II receptor is so closely related to the mouse type II receptor so as not to be immunogenic.

Production of IGF by cells in culture: The ability of cells to produce their own growth factors may have important implications for the growth of tumor cells in vivo. We previously demonstrated that rat embryo fibroblasts produce large amounts of IGF-II whereas fibroblasts cultured from postnatal animals produced mostly IGF-I, reflecting the developmental patterns of IGF-I and IGF-II in rat serum.

We have examined the IGF production pattern in fetal and postnatal human fibroblasts. IGF-I is measured by a specific radioimmunoassay, IGF-II by a radioreceptor assay, and bioactivity by a [^{3}H] thymidine incorporation assay in chick embryo fibroblasts. Compared to rat embryo fibroblasts, the levels of IGFs in medium conditioned by the human fibroblasts are 100-fold lower. There is a relatively large amount of binding protein and, under acid conditions, small binding proteins species are generated which are not easily resolved from the small molecular weight IGFs. Chemical crosslinking experiments indicate that the human fibroblasts are producing binding protein subunits analogous to the subunits from large and small IGF binding proteins of human serum.

Significance to Biomedical Research and the Program of the Institute:

The development of blocking monoclonal antibodies to the type II IGF receptor will make possible experiments to define the relative roles of the type II and type I IGF receptors. Availability of monoclonal antibodies to the type II receptor will also be of use in large scale purification of the type II receptor, elucidation of its biosynthetic pathway, and ultimately cloning and sequencing of cDNA and genomic DNA.

The understanding of the local production and action of IGFs by cells in culture may have implications for growth control in malignant growth in vivo.

Proposed Course of Research:

We will continue to attempt to develop monoclonal antibodies to the type II receptor. We will utilize an in vitro immunization method for the rat type II receptor and begin to use human type II receptor as antigen.

We will attempt to accumulate sufficient highly purified type II receptor for partial amino acid sequencing. This will enable us to have nucleotide oligmers

constructed for use in cloning a cDNA of the type II receptor.

We plan to develop monoclonal antibodies against one of the IGF binding proteins found in serum and medium conditioned by the human fibroblast cultures. These monoclonals wil be useful in the development of a radioimmunoassay to measure the binding protein in clinical samples as well as in the large scale purification of the binding protein.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04017-08 MET

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biology of the Immune Response

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David L. Nelson	Senior Investigator	MET,NCI
Laurence A. Rubin	Guest Researcher	MET,NCI
Bernard Boutin	Guest Researcher	MET,NCI
David Wagner	Medical Staff Fellow	MET,NCI
William E. Biddison	Senior Investigator	NI,NINCDS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch

SECTION

Immunophysiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	4	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☒ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Studies were undertaken to study the maturation and immunoregulation of *in vitro* T-cell and B-cell responses in normal individuals and patients with immune deficiency diseases. Patients with the Wiskott-Aldrich syndrome (WAS) and ataxia-telangiectasia (A-T) were deficient in their ability to generate cytotoxic T cells to allogeneic major histocompatibility complex (MHC) antigens *in vitro*, an observation perhaps related to the increased frequency of neoplasms observed in these patients. Antibody responses by antigen-stimulated normal B cells were shown to be macrophage and T cell dependent. Normal B cells produced mostly IgG antibodies and small amounts of IgM and IgA antibodies. This observation was due to variations in the precursor frequency for the different isotypes, and each precursor was shown to be committed to the production of a single isotype of antibody molecule. Cells from patients with the WAS and A-T produced less antibody than controls, due to defects in both T cells and B cells but not monocytes. A monoclonal antibody, termed 7G7/B6, was produced which binds to the human interleukin-2 (IL-2) receptor at a site different from IL-2 and other monoclonal antibodies (anti-Tac) against the IL-2 receptor (IL-2R). Using 7G7/B6 and anti-Tac, an enzyme-linked immunosorbent assay (ELISA) was established to quantitatively measure soluble IL-2R. Using this ELISA, a released soluble form of the IL-2R was discovered in the culture supernatants of activated human cells *in vitro*. In addition, elevated levels of soluble IL-2R were found in the serum of certain patients with cancer and acquired immunodeficiency syndrome (AIDS). Soluble IL-2Rs may play an immunoregulatory role and the level of such receptor in the serum of certain patients may be indicative of alteration in immune reactivity *in vivo*.

Project Description

Objectives:

Exposure to infectious agents and other foreign antigens elicits biologic response phenomena in host tissues which may confer protection to the host organism upon subsequent exposure to the same antigen(s). These biologic phenomena are termed immune responses and have generally been divided into two major types: 1) cell-mediated responses which require the immediate presence of immune cells for their effects to be manifest, and 2) humoral responses in which cells elaborate soluble molecules, such as antibodies, which may exert their protective effects at some distance from the immune cells which produce them. The growth and function of the cells mediating these responses are influenced by a series of polypeptide hormones called growth and/or differentiation factors, which mediate their effects by interacting with specific cellular receptors. Studies were undertaken to investigate the maturation and regulation of cellular and humoral immune responsiveness in normal individuals and in patients with a variety of immunodeficiency diseases in which congenital and acquired defects in host responsiveness are associated with an increased incidence of infection and malignant tumors. Particular emphasis was placed on studying the mechanisms underlying perturbations of immune function in these patients which might account for their increased incidence of neoplasia.

Methods Employed:

Special emphasis was placed on the study of antigen-specific cellular and humoral immune responses by human peripheral blood leukocytes *in vitro*. Two major classes of immune responses have been under investigation: 1) cell-mediated cytotoxicity responses by leukocytes whose development is thymus dependent (T cells), and 2) humoral antibody responses produced by a second class of leukocytes (B cells), whose development is bone marrow dependent and requires T cells for full maturation. Both T-cell and B-cell immune responses are highly dependent on monocytes, which are a third type of mononuclear leukocyte. *In vivo*, these cellular and humoral responses normally occur concomitantly in a highly ordered fashion with multiple cellular interactions occurring among subpopulations of T cells, subpopulations of T cells with B cells and macrophages, and B cells with macrophages. These cellular interactions result in a finely regulated response which is promptly initiated and appropriately terminated following antigenic exposure.

A major component of host responsiveness to a variety of antigens may take the form of tissue destruction (cytolysis), whereby antigen-bearing cells are lysed and eliminated. Cells capable of directly mediating such cytolytic events are termed cytotoxic effector cells. Although we have demonstrated that several types of leukocytes can function as cytotoxic killer cells, we have recently focused our attention on cytotoxic T cells which possess specific cell surface receptors for antigens expressed on those cells which undergo lytic events (target cells). Cytotoxic immune T cells are undetectable in the peripheral blood of normal nonimmunized individuals. Human cytotoxic T lymphocytes (CTL) with receptor specificities for surface antigens present on the cells of unre-

lated individuals may be generated during 7 days of in vitro culture in which density-gradient-centrifugation-prepared responder peripheral blood mononuclear leukocytes are cocultured with leukocytes from a nonrelated individual whose proliferative capacity has been inhibited by irradiation. In such cultures, responder CTL precursors proliferate and mature into cytotoxic effectors whose lytic activity is measured by a 6-hour radioisotopic release assay employing ^{51}Cr-labeled target cells from the stimulating cell donor. These immune CTL recognize a series of target cell surface molecules termed transplantation or histocompatibility antigens, which differ among individuals of the same species (alloantigens). These cytotoxic effectors are therefore termed alloimmune CTL. Alloimmune CTL probably play a major role in the host rejection of histoincompatible allografts, such as that occurring in kidney or heart transplantation, and also in graft versus host disease in which an immunoincompetent host receives a graft of immunocompetent T cells and the donor graft attacks host tissues. Since such alloimmune reactions are distinctly uncommon in nature, it has been difficult to explain these strong responses. Recent studies have suggested that alloimmune responses are reactions to foreign MHC antigens which happen to "crossreact" with self-MHC and foreign antigen (i.e., self-MHC-restricted responses). Thus, the alloimmune repertoire was probably originally generated and continues to be maintained by stimulation with a series of exogenous antigens. As such, it may represent a partial summation of the immune repertoire for self-MHC plus foreign antigens.

The second major area of emphasis centered on the assessment of humoral immune function by human peripheral leukocytes in vitro. In these studies, we concentrated on measuring specific antibody production by B lymphocytes. The presence of specific antibody in biological fluids can be detected by a variety of methods, including agglutination with particulate antigens, hemolysis employing antigen-coated erythrocytes, and radioimmunoassays. Each of these methods offers differing levels of sensitivity in detecting specific antibody. Recently, a very sensitive technique for measuring specific antibodies in the nanogram to picogram per milliter range has been described, which involves the detection of antigen-bound immunoglobulin through an enzyme-conjugated second antibody directed against the bound first antibody. This technique has been termed the Enzyme-Linked ImmunoSorbent Assay or ELISA. In a usual assay, antigen (i.e., virus) is the first allowed to bind nonspecifically in the wells of a multiwell plastic plate, and nonbound antigen is washed away. A source of putative antibody (human serum or in vitro human leukocyte culture supernatant) against the bound antigen is then added, incubated, and nonbound antibody is washed away. Next, an enzyme (alkaline-phosphatase)-conjugated heterologous (rabbit) antibody directed against human antibody is added, incubated, and unbound conjugated antibody is washed away. Finally, enzyme substrate (paranitrophenolphosphate or PNPP) is added, incubated, and the conversion of colorless PNPP to yellow paranitrophenol (PNP) product is measured in a spectrophotometer. The amount of substrate PNPP converted to product PNP is related to the amount of human antivirus antibody bound to the immobilized antigen. Such an ELISA is rapid, sensitive, and antigen specific. To assess specific anti-virus antibody production in vitro by human B lymphocytes, peripheral mononuclear leukocytes are first washed extensively with tissue culture media to remove residual traces of serum antibody adherent to the cells. Then, mononuclear leukocytes or puri-

fied subpopulations of these leukocytes (T cells, B cells) are cultured in vitro with infectious virus-containing allantoic fluid or with formalin-inactivated, zonally purified influenza viruses. The influenza viruses employed included the two antigenically distinct virus types--Type A represented by A/Hong Kong (A/Hong Kong/8/68-X-31 [H3N2]) and Type B represented by B/Hong Kong (B/Hong Kong/8/73)--and various Type A virus subtypes including A2/Aichi/2/68, MN2524 [H3N2] (A/Aichi/68), A/Udorn/72 [H3N2], A/Victoria/75 X-47 [H3N2] (A/Victoria/75), A/Bangkok/1/79 C68a [H3N2] (A/Bangkok/79), A/USSR/90/77 [H1N1] (A/USSR/77). After 12 days of in vitro culture, cumulative secretion of anti-influenza A or anti-influenza B virus antibody into culture supernatants is detected by ELISA. The antigen employed for ELISA assays included whole purified viruses of the various types and subtypes, as well as purified viral proteins such as the viral hemagglutinin and matrix protein.

In other studies to assess human B-lymphocyte maturation and function, individual B cells were isolated by limiting dilution techniques and stimulated with antigen in the presence of optimal numbers of T-helper cells and monocytes. These culture supernatants were analyzed for antibody molecules of various isotypes using either whole influenza virions or various purified viral proteins as the antigen bound to plates in an ELISA assay.

Additional studies were begun to investigate the interaction of certain leukocyte-derived polypeptide hormones (termed interleukins or growth and differentiation factors) and their cellular receptors. Such interactions lead to the growth and maturation of the cells expressing such receptors. These polypeptide hormones have been produced by the stimulation of human T cells and these molecules analyzed for effects on both T cells and B cells. Moreover, cells expressing such receptors were used as immunogens in attempts to produce monoclonal antibodies against these growth factor receptors. The molecules recognized by such monoclonal antibodies were biochemically characterized and compared with those molecules recognized by other monoclonal antibodies. Using these monoclonal antibodies, attempts were made to create "sandwich" ELISA assays to measure soluble growth factor receptors.

Major Findings:

Cytotoxic T-cell responses by human peripheral blood leukocytes in vitro: Studies were continued to define the cellular requirements for the in vitro generation of alloimmune CTL. We observed that no requirement(s) could be demonstrated in the responder cell population for T-helper cells or monocytes as long as such cells were present in the irradiated stimulator cell population. Therefore, we initiated a series of experiments to examine alloimmune proliferative and cytotoxic responses in patients with various immunodeficiency diseases. Studies have employed as stimulators either irradiated, unseparated peripheral blood mononuclear cells (PBMC), macrophage-depleted PBMC, or T-cell-depleted PBMC--the latter two populations in an attempt to uncover macrophage and T-cell defects, respectively, in the responder populations being tested. Each of five WAS patients exhibited a weak proliferative response and four of the five generated no detectable cytotoxic activity. This fifth individual also generated CTL in response to macrophage-depleted and T-cell-depleted stimulator cell

populations. Of six A-T patients studied, all mounted proliferative responses with the mean value being not significantly less than controls. Only two of the patients produced CTL activity and these patients also generated alloimmune CTL when stimulated with both monocyte-depleted and T-cell-depleted stimulator cells. These alloimmune studies confirm the existence of profound defects in the ability of A-T and WAS patients to generate immune CTL effectors, and suggest that this defect is not limited to a few antigenic specificities. Heterogeneity was observed in both patient groups with regard to alloimmune CTL generation. Among responder individuals, both T cells and monocytes were immunocompetent since responses were observed with both depleted stimulator populations. The defects in the production of immune CTL may contribute to the pathogenesis of recurrent infections and the high incidence of neoplasia in some of these patients.

Humoral immune responses by human peripheral blood leukocytes in vitro: Studies were initiated to develop methods for the assessment of specific humoral antibody production by human peripheral blood mononuclear leukocytes in vitro. Antibody molecules belong to a class of serum proteins termed the gammaglobulins. Since these gammaglobulins can confer immunity, they are often termed immunoglobulins (Ig) and several distinct subclasses of immunoglobulin (IgM, IgG, IgA, and IgE) are known to exist. Immunoglobulin-secreting cells are derived from a class of leukocytes called B cells which possess cell surface Ig as its antigen receptors. Studies in experimental animals have shown that the transition of B cells into immunoglobulin-secreting cells is antigen dependent, monocyte dependent, and for most antigens requires the positive influence of T-helper cells and is negatively regulated by T-suppressor cells.

Much of our understanding of the maturation and immunoregulation of the human humoral immune response has derived from in vitro studies employing polyclonal activators such as pokeweed mitogen (PWM) and the Epstein-Barr Virus, which activate immunoglobulin-secreting cells through receptors that are not antigen specific. We have developed a method for studying antibody production by human PBMC in vitro which is antigen induced and does not require the presence of polyclonal activators. For these studies, we have employed influenza viruses as antigens. Cultures of PBMC from < 95% of normal adult individuals produce specific anti-influenza virus antibody in vitro in the presence of Type A influenza viruses. Antibody secretion requires de novo protein synthesis, begins about day 5 of culture, and reaches maximal rates between days 5 and 7 of culture. Antibody synthesis can be induced by both live influenza Type A and B viruses as infectious allantoic fluid or formalin-inactivated, zonally purified Type A and B viruses. Antibody generation was both antigen dependent and virus specific at the induction phase since: 1) cultures in media alone or stimulated with normal allantoic fluid produced no antibody, and 2) cultures stimulated with Type A viruses produced anti-influenza A antibody but no anti-influenza B antibody and vice versa. The production of antibody was shown to require the cooperative interaction of T cells, B cells, and monocytes in culture.

Studies were undertaken to investigate the fine specificity of the human in vitro antibody response to influenza virus. Cultures of PBMC were stimulated with the following purified formalin-inactivated whole influenza viruses:

A/Aichi/68 (H3N2), A/Bangkok/79 (H3N2), A/USSR/77 (H1N1), and B/Hong Kong. Antibody production was assayed by ELISA on plates coated with each of the whole viruses, as well as on plates coated with purified viral hemagglutinin (H3 and H1) molecules. Antibody production was influenza type specific, in that cultures stimulated with B/Hong Kong made little or no antibody against any of the Type A viruses, and vice versa. There was also specificity among influenza virus subtypes--that is, cultures stimulated with A/USSR (H1N1) made only 29% as much antibody directed against A/Aichi (H3N2) as was directed against the stimulating H1N1 virus, and cultures stimulated with A/Aichi (H3N2) made only 19% as much antibody against A/USSR (H1N1) as against the homologous virus. Using the ELISA for antibody to purified virus hemagglutinin (one of two major viral surface glycoproteins and the one responsible for virus attachment to cells), antibody to hemagglutinin was demonstrated in cultures stimulated with whole viruses of H3N2 and H1N1 subtypes. Moreover, cultures stimulated with H1N1 virus produced only 10% as much antibody to purified H3 as they did to purified H1, and cultures stimulated with H3N2 virus produced only 2% as much antibody to purified H1 as they did to purified H3. Lastly, when early A/Aichi/68 (H3N2) and late A/Bangkok/78 (H3N2) strains of H3N2 virus were studied, it was observed that three of four individuals made more antibody to A/Aichi than to A/Bangkok when stimulated with either virus, perhaps reflecting the phenomena of "original antigenic sin." Fine specificity of this in vitro response was further investigated by using monospecific antibodies produced in limiting dilution cultures. Approximately 30% of the antiviral antibodies produced in these cultures bound to purified hemagglutinin, but only 7% bound to purified matrix protein. When antibodies stimulated with A/Bangkok/79 (H3N2) were analyzed for their binding to other H3N2 viruses, a variety of reactivity patterns was observed. Some antibodies were specific for A/Bangkok/79 and some bound to common determinants found on a number of H3N2 viruses. In addition, a number of antibodies were observed that did not bind to A/Bangkok/79 but did bind to earlier H3N2 viruses, demonstrating an extreme form of "original antigenic sin." A similar variety of reactivity patterns was observed when such monoclonal antibodies were tested for binding to heterotypic Type A viruses or hemagglutinins. Two major conclusions can be drawn from these in vitro studies of the B-cell repertoire to influenza virus: 1) the in vitro response faithfully recapitulates the in vivo response, and 2) unlike our previous studies demonstrating that influenza-immune cytotoxic T cells were crossreactive between subtypes (i.e., cells stimulated with H3N2 virus lyse H3N2, H0N1, and H1N1), the B-cell repertoire for influenza is largely noncrossreactive among subtypes.

In previous studies we had also shown that during respiratory infection with influenza virus, cells which spontaneously secrete IgG and IgA anti-influenza antibody are present in peripheral blood at day 6 following infection and disappear by day 27. Since secretory immune responses (particularly of the IgA class) are important in preventing influenza virus infections, we began a series of studies to examine the immunoregulation of antigen-specific IgA antibody responses by human PBMC. When stimulated in vitro with influenza A/Aichi/68 (H3N2) virus, PBMC from eight donors produced 39.1 (1.3) ng/ml (G.M. X/÷SEM) IgG and 5.0 (1.5) ng/ml of IgA antivirus antibody. Using a limiting dilution technique and Poisson analysis to calculate precursor frequencies, we found that the mean precursor frequencies of B cells producing IgM-, IgG-, and IgA-antibody

were 1:183,000, 1:148,000, and 1:1,534,000, respectively. Thus, the lower amount of IgA versus IgG antibody produced in vitro could be explained by a lower B-cell precursor frequency. To determine whether the same B-cell precursors make IgM, IgG, and/or IgA antibodies, multiple replicate wells were established to contain 1 anti-influenza virus B-cell precursor per well. In these cultures, the production of IgM, IgG, and IgA were independent, indicating that precursors make IgM, IgG, or IgA and that antigen-driven human peripheral blood B cells make only one isotype of antibody and do not undergo "isotype switching." Patients with a selective deficiency of IgA were shown to have B cells capable of making IgG and IgM antibodies, but not IgA antibodies, to influenza virus in vitro.

Studies have been initiated to utilize this method of in vitro antibody production to study maturational and immunoregulatory events in patients with immunodeficiency disease.

Specific antibody response was sought in patients with A-T and the WAS who lacked influenza-specific CTL responses. Six of seven patients with the WAS failed to produce specific antibody, as did four of five patients with A-T. Further studies were undertaken to define the cellular basis of this nonresponsiveness in certain A-T patients. One of the patients was shown to have intact monocyte function since his irradiated adherent cells reconstituted antibody responses in macrophage-depleted cultures of his MHC-matched sibling's cells. Three patients had immunocompetent B cells since their B cells produced antibody when: 1) stimulated with polyclonal B-cell activator Epstein-Barr virus, and 2) provided T-cell help in the form of normal, allogeneic, irradiated T cells. These patients' irradiated T cells, like cord blood T cells, could consistently provide allogeneic help to normal B cells. These results suggested a defect in T-helper cells as a partial cause of the immunodeficiency in A-T. However, when T cells from two patients were added to purified allogeneic B cells, both were found capable of "helping" antibody synthesis. Studies of cocultures of T cells and B cells with normal MHC-matched siblings' T cells and B cells in three cases demonstrated combined defects in T cells and B cells as the probable cause for deficient specific antibody responses in vitro in A-T patients.

Studies of the interaction of various growth and differentiation factors with their lymphocyte cell surface receptors: When resting T cells and B cells encounter exogenous antigen through their cellular receptor(s) for antigen, they undergo a transition to an activated state in which they synthesize and newly express cell-associated receptors for various growth factors. Thus, T cells activated with antigen or plant lectins express receptors for T-cell growth factor or IL-2. Using influenza-virus-stimulated T cells as an immunogen, we produced a monoclonal antibody termed 7G7/B6 which reacted with activated but not resting T cells from all donors but not Epstein-Barr-transformed, long-term B-cell lines from the same individuals. Thus, in terms of cellular distribution and its reactivity with activated T cells, 7G7/B6 appeared similar to other monoclonal antibodies against the IL-2R, such as the monoclonal antibody termed anti-Tac. However, preliminary studies demonstrated that 7G7/B6, unlike anti-Tac, had no effect on the proliferative response of IL-2R-positive T-cell lines to the growth factor IL-2. Further biochemical and functional

studies with 7G7/B6 therefore were undertaken. When the lactoperoxidase, radiolabeled cell membranes of activated normal T cells or the human T-cell leukemia-lymphoma virus (HTLV)-1-positive HUT 102B2 T cells were immunoprecipitated and subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), 7G7/B6 and anti-Tac both precipitated molecules of apparent weight of 50,000 - 55,000 daltons from normal activated T cells, and 40,000 - 45,000 daltons from HUT 102B2 cells. Pulse-chase internal radiolabeling with ^{35}S methionine followed by immunoprecipitation and SDS-PAGE analysis revealed that both monoclonal antibodies first detected a molecule of 35,000 - 37,000 daltons at 30 minutes, which subsequently matured to a 50,000 - 55,000 dalton molecule by 4 to 8 hours. Immunoprecipitation with anti-Tac identified at least one additional higher molecular weight species at approximately 110,000 daltons not observed in immunoprecipitations with 7G7/B6. Sequential immunoprecipitations with either 7G7/B6 or anti-Tac demonstrated that both monoclonal antibodies recognize the same 35,000 - 37,000 daltons and 50,000 - 55,000 dalton molecules, but only anti-Tac precipitates the higher molecular weight species. Since radiolabeled IL-2 binds to the 50,000 - 55,000 dalton molecule derived from cell surfaces reactive with anti-Tac, these studies suggested that 7G7/B6 was also directed to an epitope on the IL-2R molecule. Competition studies demonstrated that 7G7/B6 and anti-Tac bound distinct epitopes on the human IL-2R since 7G7/B6 did not block anti-Tac binding and vice versa. Studies were next performed to compare the effects of 7G7/B6 and anti-Tac on in vitro lymphocyte function. In contrast with anti-Tac, 7G7/B6 had no effect in vitro on IL-2-driven proliferation of IL-2-dependent T-cell lines or polyclonal Ig production induced by PWM. For alloimmune responses, anti-Tac but not 7G7/B6 inhibited proliferative responses and the generation of alloimmune CTL when added at culture initiation. However, cell separation studies performed after CTL generation demonstrated that the cells proliferating to alloantigen and mediating CTL effector function were 7G7/B6 positive and also anti-Tac positive. Purified IL-2 was capable of blocking the binding of anti-Tac but not 7G7/B6 to cell-associated IL-2R. Thus, 7G7/B6 is a murine-hybridoma-derived monoclonal antibody directed against the human IL-2R, and it binds to the IL-2R at an epitope distinct from that bound by anti-Tac or IL-2.

Using 7G7/B6 and anti-Tac, we were able to create a "sandwich" ELISA for the quantitative measurement of soluble IL-2R. In this ELISA the ladder of reagents from the solid phase up consisted of anti-Tac, soluble IL-2R, fluorescein-conjugated 7G7/B6, and immunoaffinity-purified alkaline-phosphatase-conjugated rabbit anti-fluorescein. The conversion of PNPP to PNP was measured spectrophotometrically, and the amount of soluble IL-2R in any particular sample related to a reference preparation of soluble IL-2R. Using this technique we were able to measure cell-associated IL-2R in detergent-solubilized extracts of cells in quantitative terms. Moreover, the measurement of cell-associated IL-2R by this technique differs from the measurement of IL-2R as detected by the binding of antibody to cell surfaces, since both 7G7/B6 and anti-Tac recognize precursor molecules not found on the cell surface which would be present in these extracts. In addition to this cell-associated IL-2R, we made the discovery of a released form of soluble IL-2R in the culture supernatants of normal cells activated in vitro. Cells activated with plant lectins, specific soluble antigens such as influenza viruses, monoclonal antibodies (OKT3) reacting with

the T-cell antigen receptor complex, and cell-associated alloantigens all released soluble IL-2R. Released soluble IL-2R was first detected 4 hours after cellular activation, reaching peak levels between days 7 and 10. The release of IL-2R was partially radioresistant, required protein synthesis, and was not simply explainable by cell death and the release of cellular IL-2R. In addition to the release of IL-2R induced in normal cells by activation stimuli, certain tumor cell lines of T-cell and B-cell lineage were found to constitutively release IL-2R. The amount of soluble IL-2R released from T-cell lines could be increased roughly 10-fold by infection of the cell lines with HTLV-1. Biochemical studies revealed that the released IL-2R had a molecular weight roughly 10,000 daltons less than the mature cell surface molecule from both normal cells and HUT 102B2 cells. In addition, the released IL-2R was also capable of binding to its ligand, IL-2. Thus, cellular activation in vitro results in the appearance of cell-associated IL-2R and also the release of a soluble form of the receptor which is capable of binding ligand and may serve an immunoregulatory function.

To examine the in vivo counterpart of this observation of the soluble IL-2R, we studied the serum of normal individuals and patients with cancers or various immune disorders for IL-2R. Normal individuals (n=33) were found to have a low, but measurable, serum IL-2R level (mean=186, range 83-335), presumably reflecting an ongoing immune activation process by various environmental antigens. Serum from patients in the United States with the HTLV-1-associated adult T-cell leukemia (ATL) had very high levels of serum IL-2R (mean=35,300, range 9,500-69,500). Similar results were obtained with sera from 21 Japanese patients with ATL. Patients with other lymphoreticular malignancies such as the Sezary syndrome (n=21), Hodgkin's disease (n=7), and chronic lymphocytic leukemia (n=20) demonstrated mean serum IL-2R levels significantly ($p < 0.001$) greater than normal. Patients with multiple myeloma (n=11), a disease of mature B cells which would not be expected to express growth factor receptors, possessed serum IL-2R which were not different from normals. In examining patients infected with other lymphotropic human retroviruses, we found that patients with HTLV-III/Lymphadenopathy Associated Virus (LAV)-related illnesses including AIDS, the lymphadenopathy syndrome (LAS), and the AIDS-related complex (ARC) all had serum IL-2R levels significantly different ($p < 0.01$) and greater than normal. In additional in vivo studies, we found that the administration of recombinant DNA-derived IL-2 to patients with cancer resulted in a significant elevation (>100-fold) in serum levels of IL-2R, perhaps blunting the therapeutic efficacy of such therapy. Patients with tumors and AIDS have variable immunodeficiencies, and various "blocking factors," capable of inhibiting normal immune responses, in their sera have been described. Soluble IL-2R, by competing with cell-associated IL-2R, may be one such blocking factor, contributing to tumor persistence and/or the immunodeficient state observed in AIDS. Thus, soluble IL-2R released into the body fluids of various patients may play an important immunoregulatory role as well as serving as an important diagnostic test, useful in monitoring the activity status of certain immune-associated diseases.

In a similar fashion, when B cells encounter exogenous antigen via Ig molecules in their cell membranes, they express cellular receptors for growth factors and

proliferate and mature into antibody-secreting cells. For these studies we have made highly purified B cells devoid of T cells and monocytes. These responder B cells are stimulated with purified anti-IgM or Staphylococcus aureus Cowan Strain A (SAC), which binds to cell-associated Ig and provides the activation signal. These activated cells then become responsive to the effects of a B-cell growth factor (BCGF) and proliferate. This activation of B cells is associated with a flux of extracellular Ca^{++} into the cells, and this Ca^{++} flux is necessary for the cells to undergo proliferation in response to BCGF. Following this proliferative response, B cells become responsive to an additional factor termed B-cell differentiation factor (BCDF), which induces these proliferating B cells to mature into antibody-secreting cells. BCGF and BCDG are both present in the supernatants of T cells stimulated with the plant lectin, phytohemmaglutinin, and also T cells stimulated with OKT3. Both OKT4-positive and OKT4-negative T-cell subsets were shown to produce BCGF in comparable amounts. Patients with hypogammaglobulinemia have low levels of circulating antibody molecules and are subject to recurrent infections and cancer. We have begun to examine the capacity of such patients to produce and respond to BCGF. Of three patients studied thus far, two have failed to proliferate following activation with SAC and BCGF, while a third patient possessed B cells which proliferated normally. An analysis of patients in this fashion will provide the necessary information as to which patients might be candidates for in vivo therapy with these lymphokines.

Proposed Course of Research:

The monoclonal antibody 7G7/B6 represents a unique reagent which is capable of identifying the human IL-2R while this receptor is occupied with its ligand, IL-2. This monoclonal antibody will be used to monitor the T cells in the peripheral blood of individuals with renal allografts, graft versus host disease, and individuals with severe combined immunodeficiency with engraftment of maternal T cells. These alloimmunized T cells should express IL-2R in vivo. The monoclonal antibody 7G7/B6 will be made available to those individuals interested in treating such patients with monoclonal antibody therapy. The monoclonal antibody 7G7/B6 will be chemically crosslinked to the monoclonal antibody OKT3 which binds to the T-cell antigen receptor complex and activates T cells for cell-mediated cytotoxicity. This complex will be used in vitro to clonally delete IL-2R-bearing antigen-reactive T cells specific for foreign antigens and alloantigens.

A wide range of studies will be performed with the ELISA for soluble IL-2R molecules to establish its usefulness as a diagnostic test and as a means of following patients with altered immune reactivity. Prospective studies are in progress to monitor the levels of serum and urine IL-2R in patients with uncomplicated renal allografts as well as patients undergoing cellular and antibody-mediated rejection phenomena. Similar studies are in progress to evaluate serum IL-2R levels in children undergoing liver transplantation. The sera of patients with various immunodeficiency diseases will be analyzed for serum IL-2R both before and after various forms of therapy. Particular attention will be paid to patients with severe combined immunodeficiency undergoing bone marrow transplantation to see if lymphocytopoiesis itself can result in increases in IL-2R levels. Studies will be continued in patients with cancer to define the usefulness of

serum IL-2R levels. Additional patient groups will be analyzed, including patients with small cell carcinoma of the lung, breast cancer, adenocarcinomas of the lung and bowel, hairy cell leukemias, and other patients with T-cell leukemias and lymphomas. Correlations will be made between the serum levels of IL-2R and the expression of cell-associated IL-R on the tumor cells themselves. Serial serum samples will be analyzed in patients undergoing various therapies to test the usefulness of this assay in monitoring responsiveness to therapy and early relapse. Additional patients with HTLV-III/LAV-related illnesses including AIDS, ARC, and LAS will be examined to determine if serum IL-2R levels can predict the outcome of infection with this virus and to what extent soluble IL-2R and treatment with IL-2 contributes to the immunodeficiency observed in these diseases. Additional nonneoplastic diseases such as rheumatoid arthritis and lupus erythematosus will be examined for serum IL-2R levels.

At a more basic level we are currently examining the mechanism(s) responsible for the release of this soluble form of the IL-2R. Studies are in progress to attempt to define if this soluble molecule is the product of the same or a different gene than that responsible for the cell-associated receptor. If the released molecule comes from the same gene, we will examine the mechanism(s) of its production.

Studies will be continued to study the lymphokines active in B-cell growth and maturation. Attempts will be made to further purify these factors and to produce monoclonal antibodies against these molecules. In addition, activated cells will be used as immunogens in attempts to raise monoclonal antibodies against the cellular receptor(s) for these lymphokines. Studies will be continued in patients with antibody deficiency syndromes in attempts to further clarify the nature of their B-cell defects in terms of these lymphokines.

Publications:

Yarchoan, R., Engler, R.J.M., Barrow, L., Kurman, C.C., and Nelson, D.L.: Antigen-stimulated human B-cell clones are committed to the production of a single class of antibody in vitro. J. Immunol. 132: 2696-2699, 1984.

Rubin, L.A., Kurman, C.C., Biddison, W.E., Brown, T., Goldman, N.A., and Nelson, D.L.: Studies of the expression of a T-cell activation antigen in primary immunodeficiency disorders and neonatal cord blood cells. In Progress in Immunodeficiency Research and Therapy I (Griscelli, C., and Vossin, J., eds.) Excerpta Medica, Amsterdam, pp. 103-108, 1984.

Yarchoan, R., Kurman, C.C., and Nelson, D.L.: Nature of the cellular abnormalities contributing to deficient in vitro specific antibody production in patients with ataxia telangiectasia. In Progress in Immunodeficiency Research and Therapy I (Griscelli, C., and Vossin, J., eds.) Excerpta Medica, Amsterdam, pp. 169-170, 1984.

Auijano, C.A., Bundy, B.M., and Nelson, D.L.: Assessment of alloimmune T-cell responses in patients with ataxia telangiectasia (AT) and the Wiskott-Aldrich syndrome (WAS): effects of varying stimulator populations and monoclonal anti-

bodies. In Progress in Immunodeficiency Research and Therapy I (Griscelli, C., and Vossin, J., eds.) Excerpta Medica, Amsterdam, pp. 109-116, 1984.

Rubin, L.A., Kurman, C.C., Biddison, W.E., Goldman, N.D., and Nelson, D.L.: A monoclonal antibody, 7G7/B6, that binds to an epitope on the human IL-2 receptor distinct from that recognized by IL-2 or anti-Tac. Hybridoma 4: 91-102, 1985.

Yarchoan, R., Barrow, L.A., Kurman, C., Strober, W., and Nelson, D.L.: Human peripheral blood mononuclear cells produce IgA anti-influenza virus antibody in a secondary in vitro antibody response. J. Immunol. (in press).

Yarchoan, R., Kurman, C.K., and Nelson, D.L.: Defective specific anti-influenza virus antibody production in vitro by lymphocytes from patients with ataxia telangiectasia. In Proceedings of the Kroc Foundation Symposium on Ataxia Telangiectasia (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04018-09 MET

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of Human Immune Defense Mechanisms and Its Control

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

PI:	Andrew V. Muchmore	Senior Investigator	MET,NCI
	R. M. Blaese	Section Head	MET,NCI
	Basil Golding	Medical Staff Fellow	MET,NCI
	Sidney Shifrin	Biochemist	DCBD,NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch

SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies are designed to explore the role of cell surface lectin-carbohydrate interactions in cellular recognition, cooperation, and regulation. Special emphasis is placed on the role of complex carbohydrates and glycoproteins in the regulation of immune response during human pregnancy. A mannose 1-6 dimer of mannose and a more complex glycoprotein have been purified from human pregnancy urine. The glycoprotein which we term uromodulin appears to specifically inhibit the action of interleukin-1. We are undertaking studies to examine the mechanism of this inhibition and the clinical significance of circulating uromodulin.

GPO 914-918

Project Description

Objectives:

Our laboratory has three long-term areas of research interest.

I. Recent evidence from numerous laboratories has confirmed and elaborated on our early studies which suggested that cellular cooperation, recognition, and regulation may in part be mediated by endogenous lectins and complex carbohydrate receptors. These early studies examined the potential role of carbohydrate recognition in assays of spontaneous monocyte-mediated cytotoxicity, antigen-specific T-cell proliferation, and Dr antigen recognition. By blocking various in vitro immunologic reactions with simple sugars, we could begin to dissect the role of carbohydrate recognition. These studies, however, were limited by the simple (but defined) nature of these blocking sugars. We have undertaken a major project aimed at purification of complex carbohydrates and glycoproteins from human pregnancy urine in an effort to characterize more complex sugars with immunoregulatory capacity. We predict that some of these purified complex saccharides will in fact represent specific receptors for endogenous lectins. We chose human pregnancy urine as a starting material because 1) previous work from our laboratory has shown the presence of immunoregulatory glycoproteins, 2) urine is a rich source of partially purified complex sugars and glycoproteins, and 3) such immunoregulatory molecules may play an important role in maintenance of the fetal allograft.

II. A second project is the development of a sensitive assay for the production of TNP-specific human antibody using TNP-brucella abortus (TNP-BA) as an antigen. These studies are examining the cellular requirements, the fine specificity, the precursor frequency, and the B-cell subsets involved in the production of specific antibody in vitro. This assay is also being used to screen the T-independent response of B cells from a variety of patients with immune deficiency.

III. We have extensively characterized a model of human spontaneous monocyte-mediated cytotoxicity. We have extended our studies with the development of cytotoxic cell lines and characterized the effect of anti-Dr antisera on this assay as well as on antigen-specific assays of T-cell proliferation. These studies have used highly purified anti-Ia antigen with both intact and Fab^2 fragments.

Methods Employed:

In vitro cellular cytotoxicity systems have been developed for antibody-dependent cellular cytotoxicity, mitogen-induced cellular cytotoxicity, cell-mediated lymopholysis, and spontaneous monocyte-mediated cytotoxicity using a sensitivity micro ^{51}Cr release assay for various target cells. These results are correlated with standard in vitro and in vivo assays of cell-mediated and humoral-mediated immunity. We have also employed standard methods of protein and carbohydrate purification, including high pressure liquid chromatography, thin layer chromatography, ion exchange chromatography, sodium dodecyl sulfate acrylamide gels, and Western blot analysis. We have also developed a sensitive ELISA assay and have used radio iodination procedures to follow our purification.

Major Findings:

I. The biochemical characterization of immunoregulatory factors found in human pregnancy urine has resulted in several major findings.

1) A low molecular weight oligosaccharide has been purified to homogeneity utilizing a combination of lectin affinity chromatography, gel permeation chromatography, and high performance thin layer chromatography. This material was screened for immunosuppressive activity using an in vitro T-cell proliferative assay to the recall antigen tetanus toxoid. In collaboration with Dr. Bo Nilson, utilizing atomic mass spectroscopy, we have deduced the structure of this oligosaccharide to be a D-mannose 1-6 D-mannopyranoside. This material is reversibly immunosuppressive at concentrations as low as 50 μM. Comparison of this urinary compound with independently purified a D-mannose D 1-6 mannopyranoside obtained from yeast cell wall mannoprotein (courtesy of Dr. Clinton Ballou, University of California, Berkeley, CA) shows that the two compounds have identical immunosuppressive characteristics; thus confirming the activity of the urinary saccharide. Addition of this mannose dimer at various times to antigen-specific proliferative assays has shown that it blocks early but not late events necessary for normal proliferation. Further characterization of the cellular requirements of this compound suggests that it requires collaboration between monocytes and lymphocytes. Thus, pretreatment of a mixture of monocytes and lymphocytes results in immunosuppression, while pretreatment of either lymphocytes or monocytes separately has absolutely no effect. Finally, we have shown that this compound has no effect on the generation of spontaneous monocyte-mediated cytotoxicity.

2) A higher molecular weight glycoprotein has also been purified using a combination of lectin affinity chromatography, gel permeation chromatography, and ion exchange chromatography. We have utilized highly purified preparations of this immunosuppressive glycoprotein to raise a rabbit heteroantiserum. Using this antiserum we have developed both a sensitive direct and direct ELISA assay. In collaboration with Dr. John McIntyre of Southern Illinois University, we have shown that our antibody conjugated to alkaline phosphatase competes in an ELISA assay with an antisera raised against purified synctiotrophoblast membranes (prepared by Dr. McIntyre). Western blot analysis has shown that these two antisera share two specificities between 69,000 and 85,000 daltons. Further Western blot analysis has shown that our antisera "sees" only these two lines when probed against crude human pregnancy urine. Our antisera does not react with human serum, and activity is not removed after extensive absorption with fresh viable human lymphocytes or immobilized human serum. Although this antisera does not demonstrate surface immunofluorescence using viable cells, it does react strongly with a membrane-associated antigen using glutaraldehyde-fixed cells. Reactivity with fixed cells is blocked by the addition of the appropriate blocking antisera. Further characterization of this membrane-associated antigen is in progress.

The bioactivity of this urinary glycoprotein has been assessed using an antigen-specific T-cell proliferative assay and the generation of spontaneous monocyte-mediated cytotoxicity. Both assays are inhibited at concentrations of less than 50 ng/ml. This urinary glycoprotein is heat labile, acid stable (pH 4.0), and nontoxic. We have expanded our studies of this glycoprotein, which we term

uromodulin. We now have several monoclonal antibodies specific for uromodulin. Enzymatic digestion demonstrates that uromodulin is rich in sialic acid and has N-linked carbohydrate. Our current data suggest that uromodulin specifically binds to cloned interleukin-1 (obtained from Dr. Peter Lomedico of Hoffman La Roche). This ability to bind to interleukin-1 also explains its in vitro bioactivity. These data represent the first description of a specific inhibitor purified to homogeneity. We have recently applied for a patent of this compound. Using our ELISA assay based on our monoclonal antibodies, we are beginning to look at the clinical significance of circulating uromodulin.

II. We have continued our interest in spontaneous monocyte cytotoxicity and the regulatory effect of antisera directed against Ia antigens. We have developed several monocytoid cell lines which spontaneously kill red blood cell targets after induction with phorbal myristate. One of these cell lines, ROHA-9, has been shown to constitutively synthesize interleukin-1. Recently, we have developed evidence that ROHA-9 also constitutively produces uromodulin. We have extended our observations with antisera directed against Ia gene products and have shown that the ability of some anti-Dr antisera to induce suppression is critically dependent upon an intact Fc piece.

III. Our third area of research involves the extensive characterization of an antigen-specific T-cell-dependent assay of B-cell function using the antigen TNP-BA developed by Dr. Basil Golding. This assay has been used to examine the cellular requirements, precursor frequency, fine specificity, and proliferative aspects of primary in vitro B-cell responses in the human. He has shown that patients with the Wiskott-Aldrich syndrome, as well as cord blood B cells, will respond in vitro to this antigen and has proposed that TNP-BA represents a prototype TI-1 antigen in the human.

Significance to Biomedical Research and the Program of the Institute:

Our studies on both monocyte-mediated cellular cytotoxicity and factor B-dependent cell-mediated cytotoxicity have had several major implications. Our data suggest that a variety of "nonspecific" in vitro killing assays are in reality quite specific and represent target-specific cell surface sugar recognition. Our phylogenetic studies imply this phenomenon is ubiquitous. We believe this type of cytotoxicity represents an important aspect of host immune defense which is independent of prior antigen exposure. Our studies with immunoregulatory compounds from human pregnancy urine offer the exciting opportunity to gain important insights into immunologic maintenance of the human placenta, as well as offering the theoretical possibility that these materials may prove to be safe, nontoxic, naturally occurring immunosuppressive agents.

Proposed Course of Research:

Our laboratory will continue to characterize the nature of carbohydrate receptors and the effect of blocking these receptors on in vitro and in vivo immune reactivity. We will extend our studies to an in vivo model in efforts to control immunologic responses in an intact animal in order to gain insight into possible approaches and therapy of diseases which are the result of overactive immune responses. We will continue to biochemically characterize the glycoprotein from

pregnancy urine. We have developed an ELISA assay which will be used to quantitate the organ and tissue distribution of this material. We also plan to develop a panel of monoclonal antibodies against regulatory glycoproteins found in human pregnancy urine.

Publications:

Muchmore, A.V., and Decker, J.M.: Uromodulin: a unique 85 kilodalton immunosuppressive glycoprotein isolated from urine of pregnant women. Science (in press).

Golding, B., Muchmore, A.V., and Blaese, R.M.: Newborn and Wiskott-Aldrich patient B cells can be activated by TNP-brucella abortus: evidence that TNP-brucella abortus behaves as a T independent type 1 antigen in humans. J. Immunol. 133: 2966-2971, 1984.

Muchmore, A.V., Decker, J.M., Blaese, R.M., and Nilsson, B.: Purification and characterization of mannose 1-6 mannose obtained from human pregnancy urine: a new immunoregulatory saccharide. J. Exp. Med. 160: 1672-1685, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04020-08 MET

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic Control of the Immune Response to Natural Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Jay A. Berzofsky	Senior Investigator	MET,NCI
	Hajime Kawamura	Visiting Fellow	MET,NCI
	Shoichi Ozaki	Visiting Fellow	MET,NCI
	Kemp B. Cease	Medical Staff Fellow	MET,NCI
	Masaharu Kojima	Visiting Fellow	MET,NCI
	Howard Streicher	Staff Fellow	LTCB,NCI
	Ira Berkower	Investigator	BB,NCDB
	John Minna	Branch Chief	NMOB,NCI
	Frank Cuttitta	Investigator	NMOB,NCI
	Charles DeLisi	Senior Investigator	LMB,NCI

LAB/BRANCH

Metabolism Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.8	4.8	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mechanisms of antigen-specific immune response (Ir) gene control and of antigen-mediated T-lymphocyte activation have been explored with the goal of understanding the regulation of the immune response and learning to manipulate it. We have found two major or immunodominant antigenic sites on myoglobin, our model protein antigen. Each is recognized with a different class II (or Ia) major histocompatibility antigen ($I\text{-}A^d$ or $I\text{-}E^d$) so that the presence of one of these Ia molecules leads to selective activation of T cells specific for one of these sites. Synthetic peptides were made which stimulate T-cell clones specific for these sites and synthetic variants used to define critical amino acid residues. For one site, critical residues were all hydrophilic and on one side of the alpha helical peptide segment, but the hydrophobic side was also necessary to be exposed, as demonstrated by studies of antigen processing. Thus, the amphipathic alpha helical structure was important. The other site was also an amphipathic alpha helix. A computer search of the sequences of six proteins with 12 known T-cell antigenic sites revealed that 10 of the 12 sites fell into regions of hydrophobic periodicity compatible with an amphipathic alpha helix, with a chance of random occurrence for each protein of $p < 0.01$. This approach may lead us to the biochemical requirements for T-cell recognition of antigen and may be a powerful tool in the search for T-cell sites and the design of synthetic vaccines. Several methods of immunopotentiation were developed. It was found that IL-2 enhanced antibody responses of low responders to the levels of high responders, perhaps by amplifying T-cell help. Also, targeting the antigen to the immune system by coupling it to anti-immunoglobulin led to enhanced uptake and presentation by B cells at low concentration, with a resultant >10-fold increase in potency for stimulation of T cells *in vitro* and >10-fold increase in immunogenicity for antibody production *in vivo*. Both of these approaches may allow development of vaccines for weak or scarce antigens, or immunization of immunodeficient patients.

Project Description

Objectives:

The critical importance of genetic factors in regulating the immune response, and especially of genes which appear to be specific for particular antigen (so-called immune response or Ir genes), has become increasingly apparent in the last 15 years. However, the mechanisms of action of these genes remain unknown. Most of the Ir genes so far described are linked to the major transplantation or histocompatibility gene complex (MHC). In the last few years it has become apparent that the mechanism of this genetic control is intimately entwined in the process by which T lymphocytes must recognize antigen in association with a major histocompatibility antigen on the surface of another cell (such as macrophage, B lymphocyte, or dendritic cell) rather than free antigen in solution. Thus, to understand Ir genes we must first determine how T cells recognize antigen plus MHC molecules, how the antigen must interact, if at all, with the MHC molecule, and what active process, if any, the cell presenting the antigen must carry out in order to mediate the interaction of antigen with MHC and with the T-cell receptor.

A second level at which the specificity of the immune response is regulated is that of idiotype-anti-idiotype interactions and networks--that is, the recognition by antibodies or immunocompetent cells of the specific receptors on other immunocompetent cells. As these receptors have unique combining sites for antigen, the combining sites serve as unique antigenic markers, called idiotypes, which distinguish those cells from others. Regulation through idiotypes and regulation by Ir genes are not completely independent (see below).

The primary goal of this research has been to define the cellular and molecular mechanisms by which these genetic and idiotypic controls regulate the immune response to natural protein antigens and, especially, how these regulate the specificity of this response. Further, we seek ways to manipulate this regulation for potential clinical application.

Methods Employed:

Myoglobins from various species were purified by the method of Hapner et al. (J. Biol. Chem. 243: 683, 1968). Fragments of myoglobin were prepared by CNBr cleavage and chromatographic purification. For antibody responses, mice were immunized i.p. with 200 μg in complete Freund's adjuvant and boosted twice with 100 μg in saline, as described previously (Berzofsky, J. Immunol. 120: 360, 1978). Myoglobin and its fragments were radiolabeled at the N-terminal alpha-amino group with $K^{14}CNO$ or N-succinimidyl-[2,3-^{3}H] propionate. Antibodies to these were measured using the polyethylene glycol precipitation direct-binding assay described previously (Berzofsky, J. Immunol. 120: 360, 1978). In competition studies other unlabeled myoglobins were added at various concentrations. The further cleavage fragments of the CNBr fragment of myoglobin, 132-153, were prepared by cleavage at Glu using Staphylococcus aureus V8 protease specific for glutamic acid (Drapeau, Methods Enzymol. XLVII: 189, 1977) or by cleavage at Tyr with N-bromosuccinimide (NBS) (Witkop and Ramachandran,

Metabolism 13: 1016, 1964). Synthetic peptides were prepared by the rapid modification (Corley et al., Biochem. Biophys. Res. Commun. 47: 1353, 1972) of the solid-phase method of Merrifield (Science 150: 178, 1965), testing for completeness of each coupling by ninhydrin analysis. S-methyl myoglobin was prepared by reaction of apomyoglobin with CH_3I to add an extra methyl group to the methionines (Jones et al., J. Biol. Chem. 251: 7452, 1979).

T-lymphocyte proliferation was studied by a method based on that of Corradin et al. (J. Immunol. 119: 1048, 1977). Briefly, nylon-wool passed T cells from draining lymph nodes of mice immunized s.c. in the tail 8 days previously with myoglobin in Freund's adjuvant were cultured for 5 days in the presence of varying concentrations of antigen (myoglobin or fragments). Proliferation was assessed from the amount of methyl-^{3}H-thymidine incorporated into DNA during the final 4 hours of culture. In some experiments, irradiated T-depleted spleen cells as a source of macrophages were pretreated with antigen in culture medium at 37°C in polypropylene tubes at 10^6 cells/0.2 ml for 1 to 24 hours, washed, and then added to macrophage-depleted T cells without soluble antigen present during the 5-day culture to assess stimulation of the T cells.

A culture system for assessment of in vitro antibody responses to myoglobin and its fragments was developed by a modification of that of Mishell and Dutton (J. Exp. Med. 126: 423, 1967). Optimum conditions were determined to be as follows: 4×10^6 spleen cells from immunized mice were cultured with 0.1 to 1.0 µg myoglobin in 1.5 ml of supplemented RPMI-1640 medium with 10% fetal calf serum in flat-bottom wells for 9 days at 37°C, 6% CO_2, on a rocking platform. On the fourth day, 1 ml of supernatant was exchanged for fresh medium, and cultures were fed daily thereafter. On the tenth to twelfth day, culture supernatants were taken to test for the presence of secreted antibody. Besides whole spleen, various cell mixtures could be cultured. For instance, spleen cells depleted of macrophages by passage over Sephadex G10 (Hodes and Singer, Eur. J. Immunol. 7: 892, 1977) were cultured alone or with macrophages from syngeneic or semisyngeneic sources. Macrophages were irradiated, glass-adherent, splenic macrophages. T cells were eliminated, where necessary, by treatment with rabbit antimouse brain antiserum plus complement. T cells were purified, where necessary, by removal of B cells and macrophages on nylon-wool columns. (See Kohno and Berzofsky, J. Immunol. 128: 2458, 1982; J. Exp. Med. 156: 1486, 1982).

Concentrations of antibodies in culture supernatants were measured by a solid-phase radiobinding assay. Wells of polyvinyl chloride flexible microtiter plates were coated by incubation with 50 µl myoglobin, 100 µg/ml for 1.5 hours. Unoccupied sites on the well were separated by a 15-minute incubation with 10% bovine serum albumin. The 50 µl aliquots of culture supernatants were incubated in the wells for 1.5 hours and unbound material washed out. Finally, ^{3}H-labeled, affinity-purified goat antibody to the Fab fragment of mouse IgG was incubated in the wells for 1.5 hours to bind to any antibody attached to the myoglobin on the plastic. All incubations were carried out at room temperature and were followed by three washes with 1% bovine serum albumin in saline. Individual wells of the plate were then cut apart and radioactivity determined by scintillation counting. Controls for nonspecific binding were all close to machine background. A standard curve with known antimyoglobulin antibody was used for quantitation (see Kohno and Berzofsky, J. Immunol. 128: 2458, 1982).

Hybridoma monoclonal antibodies specific for sperm whale myoglobin were described in a previous annual report (1980-1981). These had high affinities, between 0.2×10^9 and 2.2×10^9 M^{-1}. Their relative affinities for myoglobins of other species were determined by competitive radioimmunoassays, in which increasing concentrations of competitor were added to a constant concentration of labeled sperm whale myoglobin (7 nM) and of monoclonal antibody (on the order of 4 to 6 nM), and polyethylene glycol (Mr 6000, final concentration 10%) used to precipitate all immunoglobulin plus bound antigen. The concentration of competitor resulting in a 50% decrease in bound/free ratio for labeled myoglobin was taken as a measure of relative dissociation constant.

Rabbit anti-idiotype antibodies were prepared by immunization with 20 μg of affinity-purified monoclonal IgG_1K antimyoglobin HAL-19 in complete Freund's adjuvant. Animals were boosted after 3 and 5 weeks, and bled. Sera were absorbed six times on excess pooled normal mouse Ig attached to Sepharose, and once on MOPC 21 (also IgG_1K) attached to Sepharose, to remove antibodies to constant region determinants. These rabbit antibodies were tested for the ability to inhibit antimyoglobin binding to myoglobin-coated microtiter wells by an ELISA assay analogous to the solid-phase radioimmunoassay described above, except that polystyrene plates were used and the detecting reagent was rabbit antimouse Ig conjugated with alkaline phosphatase. The alkaline phosphatase bound to the well was determined by the hydrolysis of p-nitrophenyl phosphate to p-nitrophenolate, which absorbs at 405 nm.

To prepare syngeneic monoclonal anti-idiotypic antibodies against mouse monoclonal antimyoglobin, HAL-19, A.SW mice were immunized i.p. with HAL-19 coupled (with glutaraldehyde) to keyhole limpet hemocyanin (KLH). Spleen cells were fused with a nonproducing aminopterin-sensitive myeloma line, X63Ag8.653, and hybridomas were cloned and screened for secretion of antibodies to HAL-19 by a solid-phase ELISA similar to that above. To prevent the detecting reagent from reacting with the HAL-19 (IgG_1K) coating the plate, we used as a detecting reagent enzyme-coupled anti-mouse $IgG_{2a/2b}$. Thus, the only monoclonal obtained was IgG_2.

To establish a long-term T-cell line in culture, the procedure of Kimoto and Fathman (J. Exp. Med. 152: 759, 1980) as modified by Matis et al. (J. Immunol. 128: 2439, 1982) was used. Six B10.D2 mice were immunized in the foot pads with 100 μg of sperm whale myoglobin in complete Freund's adjuvant. Seven days later, draining inguinal and popliteal lymph nodes were removed and single cell suspensions were prepared. These were passed through a 2-g nylon-wool column equilibrated with 10% fetal calf serum in RPMI-1640, and the purified T cells were incubated at 4×10^6 cells per well in large well plates (Costar No. 3542) with 2×10^5 irradiated syngeneic spleen cells and sperm whale myoglobin at 1 μM in long-term growth medium, consisting of 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, 5 mM HEPES, and 10% decomplemented fetal calf serum. Four days later, responding T-cell blasts were harvested from the wells and purified from dead cells by Ficoll density gradient centrifugation. The T cells were then put in resting culture at 2×10^5 cells/well with 4×10^6 irradiated spleen cells for 10 days. Then 5×10^6 irradiated spleen cells plus myoglobin were added to begin

another round of stimulation and rest. After the first three or four rounds, most cells were antigen specific (alloreactivity, for example, was not detected), and the total cell population grew exponentially, about 5- to 10-fold per cycle.

The T-cell line was cloned after the sixth round of stimulation and rest. On the day before cloning, 4 x 10^6 irradiated spleen cells and fresh antigen were added to resting T cells. On the day of cloning, the stimulated T cells were counted and diluted to four cells per milliliter. The 0.1 ml of this dilution of cells was added to each microtiter well (Costar No. 3596), along with 1 x 10^6 irradiated spleen cells, more myoglobin, and 5% interleukin-2 (IL-2)-containing culture supernatant from EL4 T-lymphoma cells stimulated with phorbol myristic acetate. After 7 to 10 days, the wells showing growth were expanded into large wells (24-well plate, Costar No. 3524) with additional antigen, spleen cells, and 1% IL-2. After 1 week of further expansion, the cells were rested 1 week. These T-cell clones were expanded by rounds of stimulation and rest without IL-2, and tested for antigen specificity and H-2 restriction in the standard proliferation assay, except that 1 x 10^4 T cells were cultured with 5 x 10^5 irradiated syngeneic spleen cells in the presence of various myoglobins, or with 5 x 10^5 allogeneic or partially allogeneic spleen cells in the presence or absence of sperm whale myoglobin.

For antigen presentation and processing experiments, spleen cells were preincubated with 0.5 to 2 μM antigen at 37°C for 2 hours, washed, irradiated 3000R, and added to cloned T cells without additional antigen. Inhibitors of lysosomal function were used to pretreat the spleen cells prior to or during the pulse with antigen, as indicated.

Cleavage fragments (137-148) and (137-153) were prepared by digestion of CNBr fragment (132-153) with Staphylococcus aureus V8 protease (Pierce Chemical Co., Rockford, IL) (Drapeau, 1976), under conditions in which aspartic acid was resistant to hydrolysis so that cleavage occurred only after glutamic acid residues 136 and 148. Cleavage fragments (132-146) and (132-151) were prepared by cleavage of CNBr fragment (132-153) after tyrosine residues 146 and 151 with a 10-fold molar excess of NBS (Ramachandran and Witkop, 1967). The products of both digests were immediately isolated by reverse-phase high performance liquid chromatography (RP-HPLC) (as described below) and single, well-separated peaks were identified by amino acid analysis.

A nested series of myoglobin peptides in the region 132-146 were synthesized manually by the solid-phase method of Merrifield (1965) as modified by Corley et al. (1972). The tert-Butyloxycarbonyl (t-Boc)-protected amino acids were obtained from Vega Biochemistry, Tuscon, AZ. The synthesis was initiated at tyrosine 146 with t-Boc-2, 6-dichlorobenzyl-L-tyrosine resin (0.24 mmol/g resin) (Vega). For each residue, couplings were performed three times for 10 to 20 minutes using a 3- to 5-fold molar excess of t-Boc-L-amino acid with respect to substitution of the resin. In the case of asparagine, hydroxybenztriazole was also added at a ratio of 2 moles to 1 mole of t-Boc-L-asparagine. Small samples of resin were assayed by the ninhydrin test of Kaiser (1970) for the absence of free amino groups before proceeding to the next residue. After

coupling of each residue was complete, a portion of the peptide-resin was removed in order to generate a series of peptides, each one amino acid longer than the previous peptide at the amino terminal end. Each peptide in the series was partially deblocked and cleaved from the resin in liquid anhydrous HF-containing 10% anisole for 30 minutes at 0°C, washed with ethyl acetate, and extracted with acetic acid. Since the ε-NH_2 of lysine 140 was intentionally protected during synthesis with an HF-resistant trifluoroacetic acid (TFA) group (to allow selective modification of the ε-amino group if desired), the ε-TFA groups were removed by reaction with 1 M piperidine under N_2 at 4°C overnight (Ontjes and Anfinsen, 1969). The peptides were each purified on a Bio-Gel P4 gel filtration column (1.5 x 90 cm) in 9% formic acid followed by RP-HPLC as described below. In each case, the major peak was symmetrical and well-separated from minor contaminating error peptides. Identification and concentration were confirmed by amino acid analysis.

Synthetic peptide (132-146) was digested with carboxypeptidase-A (CPA) (diisopropyl phosphofluoridate (DFP)-treated, type II from bovine pancreas, Sigma Chemical Co., St. Louis, MO) at an enzyme-to-substrate ratio of 1:100 in 0.2 M N-ethylmorpholine acetate (NEM), pH 8.5, and the reaction terminated by lowering the pH with acetic acid (Ambler, 1972). CPA was chosen since it is known to rapidly release carboxy terminal amino acids having aromatic side chains, such as tyrosine, while only slowly releasing lysine. A pilot time course digestion was performed and monitored by RP-HPLC to establish the optimal reaction conditions for removal of Tyr 146 without releasing Lys 145. By 30 minutes, the starting material was converted to the initial product which absorbed at 214 nm but not 280 nm, an indication of the removal of tyrosine. No further change occurred over 2.5 hours, although additional products were formed after overnight digestions. The digest was then repeated on a larger scale for 1 hour. The single product was isolated by RP-HPLC and identified by amino acid analysis to be (132-145). In addition, the fact that only Tyr 146 and none of Lys 145 was released was further confirmed by reaction of the released amino acids in the digest mixture with phenylisothiocyanate and identification of the resultant phenylthiocarbamoyl (PTC) derivatives by the PICO-TAG™ procedure (Waters Associates, Milford, MA) (Bidlingmeyer et al., 1984) with only PTC-tyrosine being produced.

The CPA digestion product, synthetic peptide (132-145), was further digested with carboxypeptidase-B (CPB) (DFP-treated, Sigma) and the reaction terminated with acetic acid (Ambler, 1972). CPB was chosen in this case since it is known to release basic amino acids such as lysine much more rapidly than any other amino acids. Once again, a time course digestion monitored by RP-HPLC revealed the presence of a single new product which was evident within 5 minutes, with no additional products being formed even after 2 hours. The digest was repeated on a larger scale for 30 minutes, and a single product (132-144) was isolated by RP-HPLC and identified by amino acid analysis.

The various cleavage peptides, as well as all synthetic peptides described above, were purified and isolated by RP-HPLC using a Waters liquid chromatograph (Waters Associates). All separations were performed on a SynChropak RP-P column (250 x 4.1 mm), a 300 Å C_{18} reverse-phase support (SynChrom, Linden, IN).

The mobile phase consisted of solvent A, 0.2% (v/v) TFA (Pierce sequenal grade) in H_2O, and solvent B, 0.1% (v/v) TFA in acetonitrile (Burdick and Jackson). A linear gradient from 0 to 40% B in 60 minutes at a flow rate of 1 ml/minute was generally used. The peaks, monitored at 214 nm and 280 nm, were collected and lyophilized to remove any trace of solvent before testing in culture.

Background:

We have previously studied the in vivo serum antibody response and in vitro T-lymphocyte proliferative response to a natural protein antigen, sperm whale myoglobin, and shown that both of these responses are controlled by the same Ir genes (Berzofsky, J. Immunol. 120: 360, 1978). Using inbred strains of mice which differ only in the H-2 complex (known as congenic strains), we demonstrated that two different genes, mapping in distinct subregions (I-A and I-C) of the I region of H-2, controlled the responses to different antigenic determinants (distinct chemical sites) on the same antigen molecule--the first example of such independent control of responses to different chemical moieties on the same protein molecule. Moreover, the gene which controlled the T-cell proliferative response to a given determinant of myoglobin also controlled the production of antibodies specific for that same region of the molecule--an indication of parallel control of T-lymphocyte recognition and activation of B lymphocytes with similar specificity (Berzofsky et al., Proc. Natl. Acad. Sci. USA 76: 4046, 1979).

One level at which this Ir-gene-controlled choice of antigenic determinants recognizable is mediated is the recognition by T lymphocytes of antigen in association with cell surface structures on macrophages, which serve as antigen-presenting cells. These surface structures, which differ in different inbred strains of mice, are encoded by genes also mapping in the I region of H-2 and are, therefore, designated Ia (for I-associated) antigens. In our experiments, myoglobin-immune lymph node T cells, depleted of macrophages, from (high responder X low responder) F_1 hybrid mice were held constant as the source of responding (proliferating) T cells, and the cultures were reconstituted with liver macrophages from high responder parental mice with both Ir genes, low responder parental with neither gene, or recombinant mice with only one gene or the other. We found that the magnitude of response, and the selection of which fragments of myoglobin could stimulate these F_1 T cells in vitro, were determined by the source of the macrophages, and corresponded exactly to the response pattern of the strain of mice from which the macrophages were obtained (Richman et al., J. Immunol. 124: 619, 1980). Macrophage pretreated with antigen could also stimulate immune T cells in the absence of soluble antigen. This stimulation manifested the same Ir genetic restriction for macrophage source, and could be inhibited by inclusion of appropriate monoclonal or conventional anti-Ia antibodies, without complement, during the pretreatment with antigen (Berzofsky and Richman, J. Immunol. 126: 1898, 1981).

The limitation of T-cell proliferative responses is that they involve only T cells and macrophages, not B lymphocytes which produce antibody. In order to study the mechanisms of determininant-specific Ir gene control of the antibody response to myoglobin in vitro, we developed a modified Mishell-Dutton culture

system in which secreted antibody, specific for myoglobin, could be measured in the culture supernatants by a solid-phase radioimmunoassay. By this method, we could also measure the fine specificity of the supernatant antibodies for different fragments of myoglobin. The in vitro spleen cell antibody response to sperm whale myoglobin was found to be controlled by the same two Ir genes in I-A and I-C which controlled the in vivo response, at the level of individual determinants on different fragments of myoglobin. This in vitro antibody response was macrophage and helper T cell dependent, as shown by experiments depleting these cells and reconstituting with purified T-cell or macrophage populations. Using T cells from (high responder X low responder) F_1 hybrid mice, and B cells from F_1 or parental high or low responder strains, we found that the Ir gene control was manifested in the genetic restriction between T and B cells; that is, F_1 T cells help only high, not low, responder B cells even though adequate F_1 macrophages were added and even though the low responder B cells were adequately immunized and could be triggered by carrier-specific helpers. Even parental low and high responder T cells, from mice neonatally tolerant to both H-2 types, behaved like F_1 T cells in that they could help F_1 and high responder but not low responder B cells. Thus, both low responder T cells and low responder B cells could function, but not with each other (Kohno and Berzofsky, J. Exp. Med. 156: 791, 1982; J. Exp. Med. 156: 1486, 1982).

In order to understand the mechanism of action and specificity of these Ir genes, we have also been studying the myoglobin-specific receptors of the T cells and B cells involved in the response. As model antibodies, we have previously prepared monoclonal antimyoglobin antibodies in a high responder strain of mice. Although these had high affinity (10^8 to 10^9 M^{-1}), none of six studied in detail bound to any of the three CNBr cleavage fragments which together span the entire sequence of the protein (Berzofsky et al., J. Biol. Chem. 255: 11188, 1980; J. Biol. Chem. 257: 3189, 1982). By using a panel of 15 native myoglobins of known amino acid sequence, we could define the sites bound by three of these antibodies. All were conformation specific and two bound to assembled topographic sites consisting of amino acid residues far apart in the primary sequence but brought together on the surface by the folding of the molecule in its native three-dimensional structure (Berzofsky et al., 1980). This result was consistent with the finding that 30 to 40% of the antimyoglobin antibodies in antisera from three different species bound only to the native protein and were not removed by exhaustive affinity chromatography on any of the three CNBr cleavage fragments (Lando et al., J. Immunol. 129: 206, 1982). Compiling results from several laboratories, we found that antimyoglobin antibodies can bind to most of the protein surface (Benjamin et al., Ann. Rev. Immunol. 2: 67, 1984).

In contrast, we found the T-cell repertoire in mice to be more limited and highly skewed, and this skewing was controlled by H-2-linked Ir genes. Two high responder strains ($H\text{-}2^s$ and $H\text{-}2^d$) were found to recognize predominantly a site around residue 109, a Glu in some myoglobins and an Asp in others (Berkower et al., PNAS 79: 4723, 1982; J. Immunol. 132: 1370, 1984). This small difference between Glu and Asp could destroy crossreactivity. In contrast, another strain had a different immunodominant response pattern. Thus, immunodominance of these sites was controlled by Ir genes, which mapped to the I-A subregion of H-2

(Berkower et al., J. Immunol. 132: 1370, 1984). We prepared T-cell clones specific for myoglobin in H-2^d high responder mice and analyzed these independently for fine specificity and for the Ia histocompatibility molecule with which the myoglobin was required to be seen. Of 14 clones analyzed, five were specific for the site around Glu 109, while nine were specific for another site localized around Lys 140, as determined by using sequence variants and cleavage fragments. All of those specific for Glu 109 saw myoglobin only on antigen-resenting cells (macrophages) bearing the I-A^d Ia antigen, whereas all of those specific for Lys 140 saw myoglobin only on macrophages bearing the I-E^d Ia antigen. No cases were found of the reverse pairing of specificity and Ia "genetic restriction." This strict correlation suggests a role for these Ia antigens in selecting T cells of different specificity and may help explain Ir-gene-controlled immunodominance or skewing of the repertoire.

Current Results:

We have now confirmed the generality of this association between I-A/I-E restriction and epitope immunodominance by studying, at the population level, a new uncloned H-2^d T-cell line specific for myoglobin. The Glu 109 site was immunodominant when myoglobin was presented on B10.GD presenting cells, which express I-A^d but no I-E molecule. However, no natural recombinant exists that expresses I-E^d in the absence of I-A^d. Therefore, to do the reciprocal experiment, we used as presenting cells L cells transfected with the genes from I-E$_\beta^d$ and I-E$_\alpha^k$, a kind gift of Dr. Ronald Germain, NIAID, NIH. On B10.D2 presenting cells with both I-A^d and I-E^d, fragment 132-153 of sperm whale myoglobin, or the variant goosebeaked whale myoglobin bearing Lys 140 but not Glu 109, stimulated only about 15% of the response of whole sperm whale myoglobin. In contrast, on the transfected L cells with only I-E^d, fragment 132-153 and goosebeaked whale myoglobin stimulated 100% of the response produced by native sperm whale myoglobin. Therefore, when presented on I-E^d and not I-A^d, the site around Lys 140 is immunodominant in the bulk population. We conclude that the I-A or I-E molecule used in antigen presentation plays a major role in determining which epitopes are immunodominant.

The site bound by Lys 140-specific T cells was further mapped using cleavage and synthetic peptides to try to identify sites interacting with the T-cell receptor and any which might interact with Ia and account for the predilection for I-E^d. The 22-residue CNBr cleavage fragment, 132-153, which stimulates these clones, was further cleaved at Glu 136 or at Tyr 146, and the fragments 137-153 and 132-146 purified by HPLC. The former lost all activity, whereas the latter retained full activity. Thus, something in the stretch 132-136 was necessary, whereas 147-153 was unnecessary. The full activity of 132-146 was confirmed by solid-phase peptide synthesis, and the series of synthetic peptides 133-146, 134-146, 135-146, 136-146, and 137-146 was also prepared. All the activity was retained in the 11-residue peptide 136-146 (and the larger ones containing this), but 137-146 had lost over 90% of the activity. Thus, Glu 136 was identified as an important residue. Note that this is one turn of the alpha helix away from the other critical residue, Lys 140, in the native structure. The 11-residue peptide must contain all the information necessary not only for binding the T-cell receptor but also for interacting with Ia, if such interaction occurs.

Further studies defined other residues within this peptide that are important. Removal of Tyr 146 from synthetic 132-146 with CPA (which stops at Lys 145) and purification by HPLC showed that the peptide 132-145 retained nearly full activity, although there was some decrease in potency in titration studies. However, if Lys 145 was then removed with CPB (which cleaves Lys and stops at Ala 144) and HPLC purification, all the activity was lost. Therefore, Lys 145 is a critical residue, but Try 146 is not, although it may contribute to the potency. At the N-terminal side, we have seen that Lys 133 is not critical for activity, but a shift to the right in titration curves comparing 133-146 with 134-146 suggests that Lys 133 also contributes to potency. Therefore, we have identified a string of important hydrophilic residues, Lys 133, Glu 136, Lys 140, and Lys 145, that are approximately one turn of alpha helix apart so they are all on the same face of the helix. These residues together may form a discontinuous epitope along the hydrophilic face of the alpha helix.

The importance also of the hydrophobic side of the helix was apparent from studies of antigen processing. Proteins must be presented to T cells by a metabolically active presenting cell such as a macrophage. Having a T-cell clone which saw the same site on the native protein and a 22-residue fragment, 132-153, we asked whether both forms of the antigen required the same steps for presentation. We found that lysosomal inhibitors chloroquine and NH_4Cl, as well as the competitive protease inhibitor leupeptin, inhibited presentation of the native molecule but not that of the fragment (Streicher et al., PNAS 81: 3861, 1984). Thus, the native protein requires some lysosomal proteolytic step not required by the peptide. Since fragment differs from native in both size and conformation, we used S-methyl myoglobin, an unfolded form of the intact protein, to distinguish these. Surprisingly, the unfolded form behaved like the fragment, demonstrating that conformation, not size, is the critical difference between the fragment and native protein in determining the requirement for processing (Streicher et al., PNAS 81: 3861, 1984). Since the main overall difference between native and the other forms is that the hydrophobic residues are buried in the native protein and exposed in the peptide and unfolded form, we suggest that the purpose of the processing may be to expose critical residues, possibly hydrophobic, necessary for interaction with Ia or with the lipid membrane of the presenting cell. Examination of the structure of 132-146 in the native protein reveals a stretch of hydrophobic residues along one side of the helix which may represent this site. Thus, both the hydrophobic and the hydrophilic faces of the alpha helix of 132-146 are important for T-cell stimulation.

In general, we suggest that amphipathicity (the property of having one face hydrophobic and one hydrophilic) may be an important property for peptides which can stimulate T cells (Berzofsky, Survey of Immunol. Research, 1985), perhaps so that the hydrophobic face can interact with the presenting cell membrane or Ia while the hydrophilic face may interact with the T-cell receptor (e.g., Lys 140 and Glu 136 in our case). The myoglobin T-cell epitope around Glu 109 is also an amphipathic alpha helix. A similar example for a lysozyme T-cell epitope which supports the same concept was reported by Allen et al. (PNAS 81: 2489, 1984). The results of Godfrey et al. (Mol. Immunol. 21: 969, 1984) for presentation of tyrosine-p-azobenzenearsonate and its analogues to T cells are also consistent with this hypothesis.

These joint requirements for recognition of antigen on the surface of another cell in association with an Ia histocompatibility molecule, and the proteolytic processing or unfolding of native globular proteins which appears to be necessary for this interaction, may explain why the T-cell repertoire appears to be highly skewed compared to the antibody repertoire and why T cells do not seem to be specific for native conformation as antibodies frequently are. It also has important implications for the design of synthetic vaccines.

We have now also synthesized a nested series of peptides corresponding to residues 100-118, 102-118, 104-118, 106-118, and 108-118 of sperm whale myoglobin. Both the 100-118 and 102-118 have been purified and are active in stimulating T-cell clones specific for the immunodominant epitope around Glu 109. Thus, we have confirmed this epitope by synthesis and can begin to put some boundaries on it. The other peptides, when purified, should help to define the boundaries more precisely. This epitope is also an amphipathic alpha helix in native myoglobin.

The finding that both of the known immunodominant T-cell sites of myoglobin are amphipathic alpha helices led us to ask, in collaboration with Dr. Charles DeLisi, whether this was a general property of sites antigenic for T cells. Since T cells generally see globular proteins only after they have been cleaved into fragments by the antigen-presenting cell, the tertiary structure of the native protein should not be important for activity. Thus, it may be possible to predict T-cell antigenic sites on the basis of sequence and secondary structure alone more easily than binding to antibodies. To test this hypothesis, we determined by spectral analysis the most intense periodicity of hydrophobic residues for overlapping 7-residue segments along the sequence of six proteins for which a total of 12 T-cell antigenic sites had been reported. Ten of these 12 sites fell in regions with hydrophobic periodicity corresponding to that of an amphipathic alpha helix, with a statistical level of significance for each protein of $p < 0.01$. An eleventh site displayed a different type of amphipathicity involving sequential hydrophilic and hydrophobic blocks. Thus, T-cell antigenic sites tend to be amphipathic structures, especially amphipathic alpha helices. Although we have not yet shown the converse, that most amphipathic helices are good T-cell antigenic sites, we are currently exploring this prospect as a potentially powerful predictive tool for locating T-cell antigenic sites which may be very useful for designing synthetic vaccines.

To examine the accessibility of the antigen on the surface of the presenting cell for T-cell recognition, we have conjugated biotin to the N-terminal alpha amino group of synthetic peptide 132-146 before deblocking the ε-amino groups of lysine. After deblocking, the biotinylated peptide is active in stimulating T cells specific for this epitope. In contrast to unmodified peptide, the activity of the biotinylated peptide is blocked by avidin. The blocking is effective even if the avidin is added to the antigen-presenting cells after they have been incubated with antigen for 8 hours and then further cultured for 16 hours more to allow any uptake and processing to occur. Therefore, it is likely that avidin is blocking presentation at the cell surface, rather than blocking uptake or processing. This approach may be useful not only for identifying antigen at the cell surface, but also for characterizing surface

molecules with which the antigen interacts using the biotinylated peptide as an affinity label probe.

We have also succeeded in fusing two clones, 14.5 and 9.27, specific for the two immunodominant epitopes around Lys 140 and Glu 109, respectively, with the T-cell lymphoma BW5147 to produce T-cell hybridomas. Thus, we are able to grow these in larger quantities for isolation and characterization of the receptors. In addition, we are comparing the function and antigen response of the matched pairs of clones and hybridomas. Preliminary results indicate a lower antigen sensitivity (right shift in dose response) and a decreased sensitivity to high dose inhibition of the hybridomas compared to the clones. For the hybridomas, response can be measured by IL-2 production, but not proliferation. For the clones, we can measure both, but seem to be detecting a dissociation between the two responses, which warrants further exploration.

Ir genes: We have started two new approaches to our continuing effort to understand the action of MHC-linked and -unlinked Ir genes. A major area of controversy remaining unresolved in the mechanism of action of Ir genes is the question of whether the "defect" in the low responder is in its T-cell repertoire or in the ability of its MHC Ia antigens to interact in some appropriate way with the antigen in question. We have approached this problem using F_1 hybrid mice made by crossing high responder B10.D2 ($H\text{-}2^d$) with low responder B10.BR ($H\text{-}2^k$) mice. We had previously found that T cells from these mice preferentially responded to myoglobin on B10.D2, not B10.BR, presenting cells. However, we had been able to clone out two F_1 T-cell clones that did respond to myoglobin on the low responder presenting cells. For these clones, the low responder presenting cells presented myoglobin quite efficiently, so these cells, bearing only low responder Ia, were capable of presenting at least some part of myoglobin. To find out whether these T cells were rare clones (a quantitative T-cell repertoire problem), or whether there were discrete gaps in the ability of F_1 cells to see certain epitopes at all in the context of low responder Ia, we performed a limiting dilution Poisson analysis on a fresh population of myoglobin-immune F_1 T cells. One week after immunization, T cells from draining lymph nodes of several mice were cultured for one round of proliferation of F_1 presenting cells to adapt them to culture, and then distributed in a series of dilutions (800, 200, 40, 13, and 4 cells/well) in 60 wells per dilution on presenting cells of each of the F_1, B10.D2, and B10.BR strains. The numbers of wells with growth gave linear Poisson plots corresponding to frequencies of T cells responding to myoglobin on F_1, B10.D2, and B10.BR presenting cells of 1/112, 1/346, and 1/1149, respectively. Thus, the frequency of T cells restricted to the low responder $H\text{-}2^k$ (B10.BR) haplotype was about 3.3-fold lower than that of T cells restricted to the high responder $H\text{-}2^d$ (B10.D2) haplotype. This difference is not due to preferential expansion of $H\text{-}2^d$-restricted clones in the one round of proliferation of F_1 cells, because preliminary experiments show that the $H\text{-}2^d$-restricted and $H\text{-}2^k$-restricted clones respond equally well on F_1 presenting cells and display similar titration curves as a function of F_1 presenting cell numbers. For similar reasons, we do not think that the difference is due to greater efficacy of stimulation by $H\text{-}2^d$ than $H\text{-}2^k$ Ia molecules in the F_1 host during immunization. Rather, we believe these reflect relative differences in precursor frequency. (Unfortunately, it

is not technically possible at present to measure precursor frequency in the unimmunized animal directly.) However, to be complete, we must determine both epitope specificity and I-A versus I-E restriction for the huge number of colonies generated in this experiment, and analyze these by Poisson analysis. Although this task is not yet complete, preliminary results: 1) confirm very clearly that the 102-118 peptide is immunodominant on $I\text{-}A^d$, and the 132-146 peptide is immunodominant on $I\text{-}E^d$; 2) suggest that most of the $H\text{-}2^k$-restricted T cells are restricted to $I\text{-}E^k$ and few if any to $I\text{-}A^k$; and 3) suggests that few or none of the $H\text{-}2^k$-restricted T cells are specific for the 102-118 epitope which was immunodominant in $H\text{-}2^d$. Therefore, one very preliminary interpretation might be that the low response on $H\text{-}2^k$ presenting cells is due to the failure to see myoglobin plus $I\text{-}A^k$, in particular the major site around Glu 109, and that the small residual response is due to $I\text{-}E^k$-restricted T cells.

The second new Ir gene project is to map and study the mechanism of action of the non-H-2-linked gene (or genes), which makes strains of the "A" genetic background 5- to 10-fold higher responders than strains of the same H-2 type on the "B10" genetic background. This difference has been seen not only for myoglobin (Berzofsky, J. Immunol. 120: 360, 1978), but also for staphylococcal nuclease (Berzofsky et al., J. Exp. Med. 145: 111, 1977; Pisetsky et al., J. Exp. Med. 147: 396, 1978) and for several other antigens, including poly-(Glu, Ala, Tyr) (GAT) (Dorf et al., J. Immunol. 112: 1329, 1974) and sheep red blood cells (Silver et al., J. Exp. Med. 136: 1063, 1972). However, it is not clear whether the same gene controls all of these responses. We have compared A.SW and B10.S, which share the high responder $H\text{-}2^s$ haplotype, and also A.TH and B10.S(7R), which share a recombinant $H\text{-}2^{s/d}$ haplotype. Preliminary results of backcross analysis suggest a concordance between response to myoglobin and that to ferritin (the discordancies may be recombinants between two genes or experimental variation, and require progeny testing). So far, the results are consistent with a single gene which is unlinked to any of the markers tested so far, including H-2 (chromosome 17), Igh allotype (chromosome 12), and MUP-1 (chromosome 4). In addition, we have examined the mechanism of action of the gene by attempting to determine in which cell type it functions--T cells, B cells, or antigen-presenting cells. In mixing experiments with A.SW T cells and B10.S B cells, and macrophages or vice versa, both the B-cell source and the T-cell source seem to be important. The interpretation is complicated because both groups of cells must derive from immune mice. Therefore, if the gene acts in the presenting cell, it could affect the priming of helper T cells, and if it acts in the helper T cell, it could affect the priming of B cells. To distinguish these, further in vitro as well as in vivo adoptive transfer experiments are in progress.

Immunopotentiation: These studies have led us to discover two new methods of immunopotentiation that may be valuable for vaccine development or for immunizing with weak antigens. We studied the effect of purified IL-2 made by recombinant DNA techniques on the serum antibody response to myoglobin in high and low responder mice. The IL-2 was emulsified with the antigen in complete Freund's adjuvant to provide a sustained high local concentration. In low responder B10.BR mice, a single dose (optimum 50,000 units) resulted in a consistent 10- to 50-fold increase in specific serum antibody throughout the time

course of the response from 10 to 46 days after immunization. In contrast, no effect of IL-2 was seen in congenic high responder B10.D2 mice. With IL-2, the low responder mice achieved specific antibody levels comparable to those of high responders. The vehicle alone had no effect, and IL-2 alone, without antigen, did not induce myoglobin-specific antibody. No effect of IL-2 was seen in athymic nude mice of high responder H-2 haplotype. The effect of IL-2 may be on a small number of responding T cells in the low responder mice, but it is possible that IL-2 also acts directly on B cells in a response which remains T dependent, and therefore is not observed in athymic mice. We suggest that probably IL-2 enhances suboptimal T-cell help in the low responder, whereas help is not limiting in the high responder. This approach may enable the study of antibody responses in low responders, otherwise too weak to analyze, and may be useful in producing antibodies to poorly immunogenic antigens. Potential clinical uses include immunization with weak antigens in normal or with any antigen in certain immunodeficient patients.

Second, our studies of antigen uptake and processing by B cells and macrophages led us to examine the effect of targeting an antigen to the immune system, by covalently coupling it to anti-immunoglobulin, on its efficacy for T-cell stimulation in vitro and its immunogenicity for antibody production in vivo. In vitro, we compared the potency for stimulation of a ferritin-specific T-cell line, free ferritin, ferritin coupled to goat antimouse IgM (heavy chain specific), ferritin coupled to anti-IgG (heavy and light chain specific), or ferritin coupled to anti-IgA (heavy chain specific), and a mixture of free ferritin plus goat anti-IgG. The ferritin coupled to anti-IgM or to anti-IgG (H + L), which could bind to surface immunoglobulin of B cells, stimulated T-cell proliferation at concentrations of ferritin at least 10-fold lower than those required for the other forms of the antigen, over the entire time course of the response, with 1000R-irradiated spleen cells as presenting cells. As the goat antibodies were all of the same IgG isotype and coupling ratio, the failure of goat anti-IgA to enhance potency served as a control to exclude Fc receptor binding as the mechanism. The effect was not due to the nonspecific activation of B cells to be more efficient antigen-presenting cells, as mixtures of ferritin plus anti-IgG (H + L) had no effect, and the anti-IgG coupled to ferritin did not enhance presentation of myoglobin to a myoglobin-specific T-cell line. The enhanced presentation of ferritin conjugated to goat anti-IgG (H + L) or to anti-IgM was sensitive to radiation doses greater than 2000R and was effective at less than one-tenth the number of spleen cells, consistent with the predominance of B cells as antigen-presenting cells for this form of the antigen rather than macrophages and dendritic cells only. When B cells and accessory cells (AC) were purified from T-depleted spleen cells, only the B-cell preparation, not the AC population, manifested enhanced presentation of ferritin coupled to anti-IgG, compared to free ferritin, and was radiosensitive. Finally, allogeneic B cells could not mediate the enhancement in the presence of syngeneic splenic accessory cells (SAC); therefore, the enhancement was not due to shedding of immune complexes from B cells and subsequent presentation by SAC. We conclude that targeting the antigen to B cells as presenting cells greatly enhances its efficacy in vitro.

Similarly, at low antigen dose, the ferritin-anti-IgG (H + L) conjugate was much more immunogenic for antibody production in vivo than free ferritin, the ferritin-anti-IgA conjugate, or a mixture of ferritin plus anti-IgG (H + L). The effect was specific in that there was no increase in total serum immunoglobulin or in antimyoglobin antibodies when myoglobin was mixed with the immunogen. The lack of effect of the ferritin coupled to goat anti-IgA excludes mechanisms involving: 1) enhanced uptake by macrophages via Fc receptors or 2) help by carrier-specific T cells specific for goat IgG as the primary mechanisms. Therefore, although the data do not provide as direct evidence in vivo as in vitro, we believe enhanced B-cell presentation, as seen in vitro, is the probable mechanism of this increase in immunogenicity in vivo. If so, this may be the first demonstration of B-cell presentation of antigen in vivo, other than the indirect evidence of cognate T-B interaction. Regardless of the exact mechanism, this approach of targeting the antigen to the immune system may be of general usefulness in increasing the immunogenicity of weak antigens and synthetic vaccines.

Syngeneic monoclonal anti-idiotype: Most anti-idiotype regulation has been demonstrated with xenogeneic antisera, which is not physiologic. Very few syngeneic anti-idiotypic antibodies have been made, especially monoclonal ones. Even rarer is it to find one of these that detects a common idiotype. Also, most syngeneic monoclonal anti-idiotypes are single antibodies. We wanted to prepare a panel of such antibodies to map the idiotypic sites (idiotopes) recognized by the syngeneic immune system, as well as to try to find a syngeneic monoclonal anti-idiotypic antibody that would recognize a measurable fraction of antimyoglobin antibodies in immune serum. The latter would be useful for examining syngeneic idiotype networks.

Syngeneic monoclonal anti-idiotypic antibodies were prepared by immunizing A.SW mice with keyhole limpet hemocyanin-coupled A.SW monoclonal antimyoglobin (HAL-19, IgG_1) and screening the cloned hybridomas for production of IgG_2 binding to idiotype but not to other antimyoglobin antibodies of the same subclass in an ELISA. With these antibodies, we identified three nonoverlapping idiotopes, based on three clusters of monoclonal anti-idiotopes which mutually inhibit within each cluster, but not between clusters (Cluster I: S2, S6, S8; Cluster II: S5, S7; Cluster III: S9). Only Cluster II antibodies block the binding of myoglobin to HAL-19 and so identify binding site idiotope(s). One of these S7, but not S5, identifies an idiotope that is present on 10 to 20% of A.SW antimyoglobin antibodies in immune sera and ascites. Binding of both S5 and S7 to HAL-19 is inhibited by a rabbit anti-idiotype, which as we previously reported detects a common crossreactive antimyoglobin idiotope in immune sera (J. Exp. Med. 160: 659, 1984). The identification of a major common idiotope with syngeneic monoclonal anti-idiotypic antibodies should make possible the analysis of idiotype network regulation in vivo and in vitro in a completely syngeneic system.

Significance to Biomedical Research:

The discovery that the two immunodominant T-cell epitopes of myoglobin depend strongly on the Ia molecule involved in presenting them has several important

implications. First, it means that apparent immunogenicity of epitopes of an antigen depend strongly on the MHC of the host and cannot be predicted solely from the antigen structure. Second, it is most compatible with a direct interaction between antigen and Ia in Ir gene control. Third, the skewing of the T-cell repertoire by Ir gene effects to epitopes often different from those seen by antibodies means that to get priming of helper T cells for an anamnestic response, a synthetic vaccine should include one of these immunodominant T-cell epitopes and not just any site which can elicit antibodies. The analysis of the structure of two immunodominant T-cell epitopes and the discovery that amphipathicity, especially the ability to form an amphipathic alpha helix, is a general property of most T-cell epitopes so far described, also have important implications. First, they suggest that the antigen must interact with two structures, one hydrophilic and one hydrophobic--perhaps the T-cell receptor and either Ia or the presenting cell membrane. This information may help to model the complex ternary interaction between T cell, antigen, and Ia. Second, they suggest that one can narrow the fraction of the sequence of a protein antigen that one needs to examine to find most of the T-cell epitopes to just the amphipathic regions. Although we cannot say yet whether all amphipathic regions will be T-cell epitopes, the algorithm derived already greatly simplifies the search for T-cell epitopes and the design of synthetic vaccines. In light of what was just said about the influence of host MHC, it is already clear that, in any given host, not all amphipathic regions will be immunogenic. For instance, for lysozyme, one site is immunodominant in H-2^k, one in H-2^b, and a third site in H-2^d. The important point is that all three of these sites are amphipathic.

The Ir gene studies will hopefully shed some light on the mechanism of action of this important immunoregulatory system and allow a resolution of the current controversy on the site of action. The non-H-2-linked genetic studies may allow us to identify a new immunoregulatory mechanism of broad specificity not previously characterized.

The immunopotentiation studies may have direct clinical application to enhancement of vaccine potency, especially for weak antigens or ones in limited supply, and to immunization of certain categories of immunodeficient patients. The IL-2 may allow an amplification of limited T-cell help or directly boost the B-cell response. The anti-Ig coupling can enhance antigen presentation at low concentrations or, because it favors B-cell presentation, possibly overcome some types of macrophage deficiency. It may also be the first demonstration of B-cell antigen presentation in vivo.

Finally, the preparation of a panel of syngeneic monoclonal anti-idiotypic antibodies, and the discovery of one which recognizes a major common idiotype, may allow us to analyze idiotype network regulation in a completely syngeneic system, more physiologic than those using xenogeneic antibodies studied in the past.

Proposed Course of Research:

T-cell epitopes: We are analyzing the role of various residues in the T-cell epitopes by making substitutions at either residue already identified as important, or at others. We would like to determine whether two subsites can be defined, one which interacts with the T-cell receptor and one which interacts with Ia. The latter subsite may be identifiable by competition at the antigen-presenting cell using a peptide which has lost the former subsite. We are also, in collaboration with Dr. Charles DeLisi, developing ways to optimize the amphipathicity of a sequence, to potentially enhance the potency for T-cell stimulation over the native form. One may be able to produce heteroclitic epitopes. Conversely, a test of the amphipathicity theory would be to introduce substitutions which disrupt the amphipathicity without affecting the residues thought to be contact residues. In addition, we are testing the predictive value of the amphipathicity theory by preparing other peptides of myoglobin identified by the algorithm as having the ability to form amphipathic alpha helices. We will then test these for stimulation of uncloned and cloned immune T cells.

In immunopotentiation, we are extending the approach to targeting the antigen to the immune system by coupling myoglobin to anti-IgD. Since there is little or no IgD in the circulation to compete for binding, antigen coupled to anti-IgD should be even more efficiently targeted to B cells than antigen coupled to anti-light chain or anti-IgM. Although these other antibodies worked with adjuvant, we would like to develop an approach that does not require adjuvant so it can be used in humans.

In the anti-idiotype work, we are now looking at the effect of various monoclonal anti-idiotypes in vivo either on the induction of idiotype and/or anti-myoglobin in the absence of myoglobin, or on the response to immunization with myoglobin. We would like to detect, if present, any syngeneic physiologic idiotype networks and, if they are present, learn to perturb them to control the immune response.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04021-02 MET

PERIOD COVERED
October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Genetic Mechanisms in Human Lymphoid Neoplasms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, end institute affiliation)*

PI:	Stanley J. Korsmeyer	Senior Investigator	MET,NCI
	Ajay Bakhshi	Senior Staff Fellow	MET,NCI
	John J. Wright	Medical Staff Fellow	MET,NCI
	Katherine A. Siminovitch	Guest Researcher	MET,NCI
	Winfried Graninger	Guest Researcher	MET,NCI
	David G. Poplack	Senior Investigator	POB,NCI
	Edward Max	Senior Investigator	LI,NIAID

COOPERATING UNITS *(if any)*

LAB/BRANCH
Metablism Branch

SECTION

INSTITUTE AND LOCATION
NCI,NIH, Bethesda, MD 20205

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☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Early B-cell precursor leukemias revealed that the human immunoglobulin (Ig) genes rearrange in an ordered fashion such that heavy chain genes rearrange before light genes and κ genes generally precede λ. This ordered process includes an unanticipated deletion of the constant κ(C_κ) and κ enhancer sequence prior to λ rearrangement. We cloned three examples of the κ-deleting element (Kde) which mediates this loss and fund them to be identical. The Kde recombines site specifically with a conserved palindromic signal (CACAGTG) within the J_κ -C_κ intron and mediates all losses of κ genes on nonexpressed alleles. This is an evolutionarily conserved element that helps ensure isotypic and allelic exclusion and may mediate the ordered use of light chain genes. We exploited Ig gene and T-cell receptor gene rearrangements to detect clonal evolution from diagnosis to relapse in acute lymphoblastic leukemia (ALL) and lymphoma. Moreover, we exploited the sensitivity of this approach to identify large clonal populations in bone marrow during the presumed remission phase of ALL. We cloned the chromosomal breakpoint of the t(14;18)(q32;q21) translocation in human follicular lymphomas. The isolated 18q21 element mediates translocation in 60% of follicular lymphomas. The breakpoints cluster in a 4.0-kb region of chromosome 18 and within J_H on chromosome 14 and suggest a site-specific recombination. Our 18q21 element contains a transcriptional unit and we have cloned cDNAs from this locus. As none of the known c-onc genes map to 18q21, this provides an opportunity to characterize a new transforming gene participating in the altered growth or differentiation of the t(14;18) lymphomas.

Project Description

Objectives:

The goals of this laboratory are to identify molecular genetic events within human lymphoid neoplasms reflecting various stages of lymphoid differentiation. We focused upon the utilization of the genes encoding the antigen-specific receptors (immunoglobulin (Ig) and the T-cell antigen receptor) during the differentiation of human B cells. We established the pattern of gene rearrangement and expression within cells of certain lineage and maturation. This enabled these molecular genetic markers to make definitive insights into the cellular origin and stage of differentiation of malignancies of uncertain lineage. Such DNA rearrangements provide unique tumor-specific markers allowing the identification and serial examination of clonal cells and their progeny. By examining normal counterpart cells as well as neoplastic cells, we can discriminate between molecular events related to normal development and those unique to transformation. This approach has uncovered a sequential process of Ig gene assemblage and identified a new genetic element in the κ light chain locus that may mediate this ordered utilization. Moreover, information learned concerning the normal use of Ig genes provided the basis for defining defects in Ig gene joining or expression, responsible for the lack of Ig production or the synthesis of truncated Ig proteins in other B-cell malignancies. The Ig gene loci in certain B-cell malignancies also mediate chromosomal translocations and thus serve as sites to identify new transformation-related genes being introduced into this locus. Analysis of gene expression during discrete developmental stages of lymphoid maturation may identify specific genes related to differentiation and oncogenesis. Overall, these investigations are devoted to enhancing our understanding of the molecular mechanisms mediating differentiation and transformation.

Methods Employed:

Genomic DNA was extracted by a variety of procedures tailored to a particular type of cellular tissue examined and were designed to yield high molecular weight DNA. This DNA was then digested to completion with the appropriate restriction endonuclease, size fractionated over agarose gels, transferred to solid phase paper, and was then hybridized with randomly primed DNA probes or SP-6 RNA probes. The probes utilized were predominantly purified fragments prepared from subclones of genomic, germline clones of the coding segments of various Ig, T-cell receptor, or cellular oncogenes. Identified genes of interest within genomic DNA were then cloned from either gene machine-enriched size fractions of digested DNA or from genomic libraries prepared from such cells. Standard screening isolated phage plaques with the desired inserts and routine procedures of plasmid subcloning, restriction endonuclease mapping, and DNA sequence analysis by Maxim-Gilbert or M13 dideoxy termination methods followed.

RNA for analysis was prepared from fresh viable cells. A guanidine thiocyanate procedure was used to prepare total RNA, and nuclear and cytoplasmic fractions were also prepared at times. Poly A-selected RNA was selected over oligo-dT-

cellulose columns. Such RNAs were analyzed by Northern analysis using formaldehyde gels or were assessed by dot blot quantitation.

Total and subtraction-enriched cDNA libraries were prepared by standard methods utilizing oligo-dT priming and reverse transcriptase for the first strand synthesis. A second strand was generated by dC tailing of the first strand and oligo-dG priming for the second strand. Such dscDNAs were dC tailed and annealed to a dG-tailed PBR Pst vector prior to transformation of DH-1 cells. Alternatively, these dscDNAs were internally protected with EcoRI methylase, EcoRI linkers were ligated and digested, and the cDNA size separated on Seph CL4B columns. Such EcoRI-linkered cDNAs were inserted into the CI site of λgt10 and subsequently screened. Subtractive hybridizations were performed with certain first strand cDNAs and other poly A RNAs utilizing a phosphate hybridization buffer and subsequent hydroxyapetite column separation of double- and single-stranded populations. Such subtraction-enriched cDNAs could either be utilized as probes or as substrate for generating cDNA libraries. In some experiments an oligonucleotide primer corresponding to portions of the CH_1 domain of C_μ was utilized with reverse transcriptase in order to define the 5' extent of the mRNA encoding C_μ by primer extension analysis. Such oligonucleotide-primed first strands were at times used to generate enriched dscDNAs that were directly cloned into M13 phage for sequence analysis.

Analytic flow microfluorometry with a fluorescence-activated cell sorter was used to assess the presence of various monoclonal and heteroantibody defined cell surface antigens. This included at times dual fluorescence studies utilizing both Texas Red and fluorescein to assess the simultaneous presence of cell surface antigens. Surface antigens were also characterized by techniques of immunoprecipitation using either protein A or a second antibody following initial preclearing. The final washed pellets were reduced and analyzed on SDS polyacrylamide gels. Cytoplasmic and secreted Igs were measured by a double antibody radioimmunoassay sensitive to the picogram range. Routine procedures of cell separation including sheep red blood cell rosette separation antigen-antibody complex plates, anti-Ig columns, and monoclonal antibodies plus complement were used.

Major Findings:

Humans utilize both light chain gene classes appreciably, using κ 60% and λ 40% of the time. Unexpectedly, however, our examination of mature human B-cell lines and leukemias revealed a marked dichotomy in the excluded L-chain isotype in κ- versus λ-producing B cells. κ-producing B cells usually retained germline λ genes, whereas λ-producing B cells had no remaining germline κ genes, having either rearranged or, more frequently, deleted them. In fact, this surprising loss of κ genes in λ B cells was a normal developmental event since normal, nontransformed λ-bearing B cells from the peripheral blood of a normal individual also displayed deletions of κ genes. An examination of L-chain genes within B-cell precursors at earlier stages of differentiation revealed seven instances in which κ gene deletions had occurred prior to λ gene rearrangements. This indicated that the κ deletional rearrangements were a

prospective event and thus suggested that they might influence L-chain isotype selection in B cells.

Our further characterization of this deletional process indicated that, while the C_κ and κ enhancer elements were uniformly deleted, the J_κ region was deleted in 25% of the cases but was present in a rearranged form 75% of the time. We reasoned that these rearranged J_κ segments which lacked C_κ would provide a necessary handle to identify and clone the 3' located recombinatorial element responsible for this event. Three such alleles from pre-B cells were molecularly cloned by screening genomic libraries constructed from these cells with a J_κ probe. All three alleles had undergone both a 5' rearrangement event (presumably attempted V/J joining) and a 3' rearrangement which introduced a new sequence. Restriction map analysis revealed that all three of the isolated κ-deleting recombinatorial elements were identical. DNA sequence analysis confirmed this identity. Furthermore, the recombinatorial breakpoint is focused within a limited region in the J_κ-C_κ intron. This prompted a further subcloning and sequence analysis of this region which identified the palindromic signal (CACAGTG) within the J_κ-C_κ intron at the site of rearrangement for the Kde. Since this same heptamer also flanks germline V and J regions, the recombinatorial enzymes that help activate this gene (V/J joining) may also mediate its destruction by rearranging the Kde. When a unique portion of the Kde was used as a probe to characterize a panel of somatic cell hybrids, the Kde was shown to be located on human chromosome 2. Moreover, this same probe revealed that the Kde uniformly mediated all the deletions of κ genes observed in λ-producing B cells. The Kde was also responsible for all losses of κ genes at the pre-B-cell stage and even for the deletion of the excluded κ gene allele in κ-producing B cells. In contrast, the Kde remained in its germline form on all successful κ-producing alleles. Moreover, κ gene loss is an evolutionarily conserved event as the Kde has a murine homologue in the RS sequence described by Durdick, Selsing, and their coworkers. We have compared the mouse and human Kdes by heteroduplex, hybridization, and sequence analysis, and have found them to be highly conserved. The uniformity of this event and its evolutionary conservation argues that this element helps ensure isotypic and allelic exclusion of light chain genes and could well play a major role in the ordered use of light chain genes.

We had shown that the non-T, non-B form of ALL was a developmental series of B-cell precursors. These pre-B leukemias revealed a cascade of Ig gene rearrangements in which heavy preceded light and κ rearranged before λ. We have now determined that a coordinate sequence of cell surface antigen expression exists that is synchronous with the hierarchy of Ig gene rearrangements. The very earliest identifiable B-cell precursors displayed H-chain gene rearrangements, HLA-DR, and the p40/80 B_4 heterodimer described by Nadler *et al*. Later in development, pre-B cells add the common acute lymphoblastic leukemic antigen (CALLA) and subsequently rearrange κ and/or λ L-chain genes. The most mature B-cell precursors frequently display the pp35 B_1 molecule, but its time of onset is variable. Correspondingly, normal counterparts of these pre-B-cell stages can be identified within fetal and adult bone marrow by dual fluorescence studies. Thus, the B-cell precursor leukemias represent clonal expansions of apparently normal stages of development that provide a source of

cells to further define genetic events in early B-cell maturation. This has been approached by quantitating and isolating the genes which are transcriptionally activated during these pre-B-cell stages. Procedurally, subtractive cDNA libraries were compared by taking the total cDNA from the most mature pre-B-cell stage and hybridizing it with the poly A RNA from the least mature pre-B cell to remove the shared messages. Such an enriched library is then screened with a more rigorously subtracted probe generated from similar cells. The isolated clones are enriched for even low frequency messages which are transcriptionally initiated during this window of early B-cell lineage commitment.

Together with Dr. Edward Max, we have also examined the developmental time of expression of the 15,000 dalton protein known as J chain which is ultimately responsible for linking IgM and IgA pentamers. Utilizing a human genomic clone of the J chain gene, we found that J chain was expressed as early as the pre-B-cell stage in humans but was not uniformly present in pre-B cells, appearing in our sample only in those cells with cytoplasmic μ. Of the 10 lines we examined that were representative of mature B cells, eight, including all IgM or IgA secretors, contained J chain RNA. The correlation of J expression with heavy chain isotype was not absolute, as a γ,κ cell line continued to express J chain while none was apparent in the μ,λ cells of a chronic lymphocytic leukemia. Cells not in the B-cell lineage did not express J chain. Thus, J chain expression may prove to be biphasic with initiation early in the pre-B-cell series with loss of expression at a time when only surface μ is produced, and resumption during mature B-cell and plasma cell stages despite which isotype is produced. Thus, the availability of a human J chain clone allows the regulation of this gene during B-cell differentiation to be determined.

Since presentations of pre-B ALL represent stages within a coordinate sequence of Ig gene rearrangements and surface antigen expression, we wished to examine the natural history of this leukemia to determine if genetic progression occurred over time. However, none of the cell surface antigens were tumor-specific clonal markers as they existed on normal cellular analogs as well. Consequently, we turned to the DNA rearrangements of Ig genes as tumor-specific markers unique to each neoplasm that allowed us to follow their genetic history. We discovered that individual cases are not static but can undergo genetic progressions from diagnosis to relapse. There are normal developmental progressions which occur in pre-B cells such as intermediate (D/J) to complete (V/D/J) rearrangements or movements from H-chain to L-chain rearrangements. Other common events include unanticipated deletion of a H-chain allele. While it is theoretically possible that leukemias at diagnosis and relapse could be truly biclonal, resulting from totally separate transformation events, all cases were incompatible with that explanation. Even those leukemias which displayed varying Ig gene rearrangements shared at least one common molecular marker. This indicates that, while pre-B ALL is an early neoplasm, the progenitor cell appears to be committed at the Ig gene rearrangement level rather than being pluripotential.

Since specific Ig gene rearrangements are only detectable in a clonal expansion of cells, we exploited this as a sensitive molecular marker to search for per-

sistent clones following therapy and to identify the recurrence of clonal populations prior to histopathologic evidence of relapse. Pre-B ALL patients studied during early induction phases showed the expected correlation between the presence of clonal cells and histopathologic evidence of lymphoblasts. Of 45 bone marrow aspirates well into remission phases, a full 42 had no evidence of residual disease at a sensitivity of 1-5%. Three patients were exceptional, demonstrating large clonal populations of clonal cells during remission phases despite the lack of lymphoblasts. These clonal cells were noted 6 months and 9 months prior to relapse, and to date these are the only two individuals who relapsed. This indicates that what is regarded as remission in lymphoid neoplasms is not always an absence of clonal cells. Whatever the origin of such cells, they are under a markedly different regulatory control than the leukemic cells at diagnosis or relapse. The fact that the only patients with clonal cells relapsed raises the possibility that the molecular demonstration of clonality may facilitate the early detection of individuals who will ultimately relapse.

All mature B cells invariably display rearrangements of their Ig H-chain genes and their L-chain genes, while cells of other hematopoietic lineages tend to retain germline Ig genes. L-chain genes have uniformly been retained in their germline form within T cells, as well as myeloid, monocytic, histiocytic, and promyelocytic cell lines and leukemias. These non-B-cell lineages usually retain germline H-chain genes as well, although occasional T cells or myeloid cells will display H-chain gene rearrangements. Thus, the detection of simultaneously rearranged heavy plus light chain genes within lymphoid neoplasms serves as a marker for B-cell lineage commitment.

Similarly, the genes of the antigen-specific receptor for T cells are also proving useful in placing DNA level markers of lineage commitment and clonality within the T-cell lineage. The β chain gene of the T-cell receptor rearranges in all mature T cells and only occasionally in the B-cell lineage. Utilizing both the Ig and the T-cell receptor gene probes has enabled us to detect monoclonal B-cell malignancies within lymphomatous lymph nodes in which infiltrating, polyclonal T cells were actually predominant. Examination of the t(4;11)(q21;q23) acute leukemias has revealed that they possess Ig gene rearrangements of a pre-B-cell stage but also retain some monocytic characteristics. We are extending this information on their lineage specificity in an attempt to characterize the chromosomal breakpoint in these cells. We are pursuing the thesis that one side of a malignancy-associated chromosomal translocation will contain a transforming gene while the other side will be a phenotypic landmark gene pivotal to that stage of cellular maturation. In collaboration with Dr. John Kersey, we are exploring the relationship of the c-ets gene complex at 11q23 with the J chain gene on chromosome 4 (pre-B marker) and an interferon-induced gene at 4q21 (monocyte gene). Characterization of the genetic lineage may thus provide insights into the transformation events associated with each developmental stage of malignancy.

An additional rearrangement may affect the Ig gene loci within certain B-cell malignancies and introduce non-Ig gene information from a separate chromosome. We have characterized the t(14;18)(q32;q21) translocation that occurs in 60-80%

of follicular lymphomas and some diffuse lymphomas. Examination of DNA from tissues and cell lines of t(14;18)-bearing lymphomas revealed that SU-DHL-6 possessed an Ig H-chain gene rearrangement that could not be explained by the normal developmental events of variable region gene assemblage or H-chain class switching. Specifically, the J_H region had been divided on the phenotypically excluded allele that was not responsible for Ig production. We molecularly cloned the Ig alleles from this cell and exploited the unexpected J_H gene rearrangement to identify the chromosomal breakpoint. We characterized a 21.6-kb fragment from the derivative 14 chromosome which had C_γ switch and the enhancer region juxtaposed with some 10.5 kb of foreign DNA information. This was proven to be of chromosome segment 18q21 origin by examining somatic cell hybrids and by chromosomal in situ hybridization. Significantly, the juncture of chromosome 18 with 14 is flush with the 5' end of the J_H6 coding segment. The heptamer and nonamer which flank each J_H region are missing. This suggests that the same signals and recombinase that mediate V/D/J rearrangements may also be responsible for this site-specific recombination between chromosomes.

Having cloned one t(14;18) breakpoint, we wished to determine how frequently this region of 14 and 18 was mediating translocations in other B-cell tumors. The breakpoints on chromosome segment 18q21 have proven to be remarkably focused. All four cell lines we had which bear the t(14;18) translocation broke within a small 4.3-kb breakpoint cluster region on chromosome 18. Similarly, the breaks are restricted to the J_H region or its immediate 5' flanking sequence on chromosome 14. In fact, J_H region information and our isolated 18q21 element can be placed on the same small restriction fragment in all instances where we can define the 18 rearrangement. This enables the discrimination of the productive Ig gene on the normal chromosome 14 from the excluded and translocated Ig gene on the derivative 14. These translocation breakpoints are not in vitro phenomenon, but can be detected in fresh lymphoma biopsy tissues. Of 15 unselected follicular lymphoma biopsies, eight displayed rearrangements (60%). Moreover, 10 of the 12 definable breakpoints clustered within the small 4.3-kb region of chromosome 18. In contrast, the examination of other neoplasms indicates that the 18q21 element does not appear to normally rearrange during B-cell differentiation or within other cellular lineages. Since this rearrangement is restricted to lymphomas with proven or expected t(14;18) translocations, it serves as a molecular marker specific for a translocation and presumably a transformation event. Because the site of the breakpoint varies from tumor to tumor, this has enabled us to follow the natural history of follicular lymphoma. We have found that, while the Ig gene configuration on the normal chromosomes can vary, the rearrangement depicting the chromosomal translocation remains fixed over time. This unequivocally demonstrates that these tumors are not truly biclonal but share a common transformation event. Instead, they undergo a clonal evolution which is reflected in a genetic variation in Ig gene rearrangements. This 18q21 element has also been pivotal in determining the chromosomal origin of the translocated partner in translocation in which the reciprocal partner is indeterminant by routine cytogenetics.

By analogy with c-myc in Burkitt's lymphoma, chromosome 18 may be contributing a transforming gene whose expression is disturbed by its new location within the Ig locus. We have identified a transcriptional unit within the 18q21 element we cloned. This region recognizes a 6.5-kb and a 4.0-kb mRNA species within t(14;18) lymphomas. We have also isolated cDNA clones from a λgt10 library we constructed from one such lymphoma which corresponds to these mRNAs. Thus far, none of the identified cellular oncogenes are known to map to chromosome segment 18q21. Furthermore, our 18q21 encoded gene has no close homology with any of the available v-onc or c-onc genes. It is important to emphasize the differences between the t(14;18) and the t(8;14) breaks of Burkitt's lymphoma. The chromosomal breakpoint may prove to be more clustered on both chromosomes 14 and 18 in follicular lymphomas than was the case in Burkitt's lymphoma. Also, the characteristic breakpoint within J_H would retain the enhancer region in close proximity to the 18q21 gene introduced onto the derivative 14. Cloning the chromosomal region that mediates the t(14;18) has provided the opportunity to study a potentially new transforming gene that is perhaps involved in the disordered growth or differentiation of this B-cell lymphoma.

Proposed Course of Research:

We will determine the physiologic role for the Kde during B-cell differentiation. Is its sole role to delete the κ locus and thus ensure a high percentage use of λ? Alternatively, does it encode a transacting factor that affects λ gene rearrangement or expression? We are searching for a transcriptional unit within the germline or rearranged form of this gene. We propose to isolate cDNAs from any active locus and generate antibodies against any predicted protein product. These would be used to purify, localize, and determine the developmental expression and action of such a product. We will look for recombinatorial signals (heptamers and nonamers) at the 5' end of the germline clones of the Kde. We will determine if effective V_K/J_K rearrangements are located upstream to the rearranged Kde and whether their promoter influences the Kde. Similarly, we will determine if V_K regions serve as the target site for Kde rearrangements that land 5' of J_K.

We will continue to utilize rearrangements of Ig and the α, β, and γ T-cell receptor genes as molecular markers of clonality, lineage, differentiation, and translocation. We will determine whether the detection of clonal populations during remission phases of ALL can predict patients who will ultimately relapse. Furthermore, the capacity to place multiple clonal markers, especially those of translocations, enables us to follow the genetic history of these neoplasms.

We will also attempt to determine the identity and function of the gene encoded by the 18q21 element in t(14;18) lymphomas. The multiple cDNAs from this locus are being mapped, heteroduplexed with genomic clones, and sequenced. We will prepare hetero- and monoclonal antibodies against synthetic peptides as well as expression vector-produced protein products. These will be used to determine the biochemical characteristics and cellular localization of this product. Combinations of protein and RNA expression studies will determine the expres-

sion of this product within translocated cells, resting normal B cells, activated B cells, and any cell cycle dependence. We will examine the alteration of its expression in the translocated versus the germline form and determine if the Ig enhancer region plays a role. In gene complementation studies we will examine whether the 18q21 gene can complement other oncogenes and result in the transformation of rat embryo fibroblasts. We will also characterize its expression in other cellular lineages and from all of this information look for its normal role in B cells as well as its altered role in neoplasms. We have also cloned another set of reciprocal chromosomal breakpoints (derivatives 18 and 14) from the same cell and will compare these breaks with the germline 14q32 and 18q21 region. This will determine, base for base, the site of recombination and confirm whether this is always a site-specific mechanism. Moreover, we will characterize the germline breakpoint cluster region on chromosome 18 to see if recombinational signals (heptamers, spacers, nonamers) are located there and what their normal physiologic role might be. By analogy with our t(14;18) breaks and the Burkitt t(8;14) breaks, we suspect that chromosomal translocations associated with malignancies represent a human gene map. At one side of the break may be a phenotypic landmark gene pivotal to that stage of development, while the other side contributes a transforming gene. We are exploring other breakpoints such as the t(4;11) leukemia translocation and a constitutional t(9;14) in T-cell neoplasms in an attempt to identify previously unknown genes. These approaches will provide further insights into the molecular genetic basis of human lymphoid development and its associated neoplasms.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04022-02 MET

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Human Interleukin-2 Receptor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Warner C. Greene	Senior Investigator	MET, NCI
OTHER:	Warren J. Leonard	Senior Staff Fellow	MET, NCI
	Joel M. Depper	Expert	MET, NCI
	Martin Krönke	Guest Worker	MET, NCI
	Richard J. Klausner	Chief, Cellular Biology Section	MET, NICHD
	Thomas A. Waldmann	Chief	MET, NCI

COOPERATING UNITS *(if any)*

Richard J. Robb, Ph.D., Principal Investigator,
E.I. duPont de Nemours, Glenolden, Pennsylvania
Ellen Vitetta, Professor of Microbiology, University of Texas
Southwestern Medical School, Dallas, Texas

LAB/BRANCH

Metabolism Branch

SECTION

INSTITUTE AND LOCATION

Division of Cancer Biology and Diagnosis, NCI, NIH

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4 1/2		

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

cDNAs encoding the human receptor for interleukin-2 (IL-2, T-cell growth factor) have been molecularly cloned and the receptor protein biochemically characterized. The human genome contains a single receptor gene located on chromosome 10p band 14-15 and organized within eight separate exons. Transcription of this receptor gene results in three differently sized classes of mRNA varying in length due to the use of at least three different polyadenylation signals. Alternate splicing may also occur which results in removal of a 216 base pair segment corresponding to exon 4. This aberrantly spliced mRNA does not appear to encode a protein capable of binding IL-2 or anti-Tac. The mature IL-2 receptor protein is composed of 251 amino acids. This peptide (M_r 33,000) is cotranslationally modified by addition of N-linked carbohydrate producing two precursor forms (M_r 35,000 and 37,000). These precursors are subsequently exported to the Golgi apparatus where O-linked sugar, sialic acid, and sulfate are added prior to display of the mature receptors (M_r 55,000) on the cell surface. T-cell activation is characterized by an early rise and later fall in IL-2 receptor expression which is involved in the regulation of the T-cell immune response. The variable expression of IL-2 receptors is controlled, at least in part, at the level of receptor gene transcription. IL-2 receptors can be reinduced in "senescent" activated T cells, which have lost >90% of their receptors, by stimulation with antigen or mitogen, agents which activate protein kinase C (PMA, phospholipase C, diacylglycerol congeners) and IL-2. Human T-cell leukemia-lymphoma virus I (HTLV-I)-infected adult T-cell leukemia (ATL) cells uniformly express large numbers of IL-2 receptors. The amplified expression of receptors in ATL cells involves constitutive transcription of the IL-2 receptor gene and use of three rather than two promoters. Agents which activate normal IL-2 receptor gene transcription may selectively inhibit receptor transcription in ATL cells. ATL cells can be selectively killed with anti-IL-2 receptor antibodies coupled to the toxic A chain of ricin.

GPO 914-918

Project Description

Objectives:

The principal objectives of these studies were: 1) to clone, sequence, and express cDNAs encoding the human interleukin-2 (IL-2) receptor; 2) to define the organization, location, and sequence of the normal IL-2 receptor gene; 3) to biochemically characterize this receptor using monoclonal anti-Tac, an anti-IL-2 receptor antibody; 4) to study IL-2 receptor gene expression in normal and neoplastic T cells; 5) to evaluate potential use of anti-Tac as a biological modifier of the human immune response; and 6) to examine tumor-specific cytotoxic effects of anti-Tac conjugated to the A chain of ricin employing cell lines from patients with human T-cell leukemia-lymphoma virus I (HTLV-I)-induced adult T-cell leukemia (ATL).

Methods Employed:

1) Cloning of cDNAs encoding the human IL-2 receptor: The IL-2 receptor was purified to near homogeneity from HTLV-infected HUT 102B2 cells using anti-Tac as an immunoaffinity support. These receptor preparations were then used to determine the N-terminal amino acid sequence using gas phase micro-sequencing techniques. As well, purified receptor was used to immunize a rabbit to raise a high titer monospecific heteroantibody. RNA was isolated from HUT 102B2 cells by cesium chloride isopycnic centrifugation in the presence of guanidine thiocyanate. mRNA was prepared by oligo dT cellulose affinity chromatography. This mRNA was used to construct a cDNA library in lambda gt10 bacteriophage. This library contained 2.4 million recombinant phages containing inserts ranging in size from 500 base pairs to several kilobases. This library was then screened with a synthetic oligonucleotide probe (17 mer) prepared based on the N-terminal amino acid sequence. Eleven candidate clones for the IL-2 receptor were identified and cDNA inserts from three of these clones were subcloned into pBR322 and M13 bacteriophage. Each of the candidate clones was then tested for the capacity to selectively hybridize to mRNA which when translated in a cell-free wheat germ lysate translation system and precipitated with the anti-IL-2 receptor heteroantibody would yield primary translation product for this receptor. In addition, the DNA sequence of each of the three cDNA clones (0.9 kb, 1.7 kb, and 2.4 kb) was determined using the dideoxy chain termination method of Sanger. All sequences were analyzed on an IBM 370 computer using the program of Queen and Korn. These cDNA clones were also used to probe genomic blots of restricted human DNA to evaluate the number of genes encoding the IL-2 receptor. Similarly, mRNA encoding the human IL-2 receptor was studied by Northern blot analysis. All ^{32}P probes were prepared by nick translation or random priming of purified cDNA inserts or intact plasmids. cDNA clones were also expressed using the eukaryotic expression vector, pcEXV-1. This plasmid contains the SV40 promoter and enhancer elements 5' to an EcoRl cloning site with a distal polyadenylation signal. Following subcloning of the cDNAs into this vector, determination of insert orientation, and large-scale preparation of the appropriate plasmids, DNA was transfected by $CaPO_4$ precipitation into COS-1 cells. Expression of functional IL-2 receptors was measured 72 hours later in binding assays using radiolabeled IL-2 and anti-Tac.

2) Studies of IL-2 receptor gene organization, sequence, and location: Utilizing IL-2 receptor cDNA probes, overlapping phage clones were isolated from two genomic DNA libraries. These clones were mapped with restriction enzymes and the DNA sequence of each of the exons and intron-exon junctions was determined. The promoter region of the IL-2 receptor gene was studied using primer extension analysis and S1 nuclease protection assays. Activity of the putative promoter fragment was studied by ligation into JYMCAT-0 plasmid followed by transient expression of this construct in JURKAT cells and measurement of resultant chloramphenicol-acetyl transferase activity. The IL-2 receptor gene was localized by in situ hybridization utilizing ^{3}H-labeled IL-2 receptor cDNA probes prepared by nick translation.

3) Regulation of IL-2 receptor gene transcription in normal and leukemic T cells: RNA was isolated from varying cell types following stimulation and analyzed by Northern blotting and in S1 nuclease protection assays. To directly study transcriptional control of IL-2 receptor gene expression, nuclear runoff assays were performed with nuclei from unstimulated and stimulated normal and leukemic T cells. Post-transcriptional processing of IL-2 receptor mRNA, including utilization of different polyadenylation sites and aberrant post-transcriptional splicing, were evaluated in S1 nuclease protection assays.

4) Stable expression of IL-2 receptor cDNA: To achieve long-term cell lines stably expressing IL-2 receptors, thymidine kinase deficient murine L cells were cotransfected with an SV40 expression vector containing a full-length IL-2 receptor cDNA and a plasmid containing the thymidine kinase gene. Following HAT selection, growing colonies were isolated and screened for IL-2 receptor expression, IL-2 responsiveness, and affinity of receptors displayed for radiolabeled IL-2.

5) Biochemical characterization of the human IL-2 receptor: As a source of human cells expressing IL-2 receptors, short- and long-term human T-cell lines were established in conditioned medium containing IL-2. HTLV-I-infected T-cell lines were also established and carried in long-term culture. Purification and characterization of the IL-2 receptors involved biosynthetic labeling of activated T cells with varying isotopes (^{35}S-methionine, ^{3}H-D-glucosamine, ^{32}P-orthophosphoric acid, and ^{35}S-sulfuric acid), or surface iodination with lactoperoxidase, immunoprecipitation of labeled receptors with anti-Tac, and analysis by one- and two-dimensional SDS-PAGE. Covalent crosslinking of purified IL-2 to its radiolabeled membrane receptors was performed using disuccinimidyl suberate (DSS). Post-translational modification of the receptor was studied using pulse-chase labeling, tunicamycin blockade of N-linked glycosylation, and digestion with endoglycosidase F and neuraminidase. Golgi-associated processing events were identified using the carboxylic ionophore, monensin.

6) Measurement of IL-2 receptor number and affinity in varying neoplastic, immunodeficient, and normal cell populations: To quantitate IL-2 receptor expression, monoclonal anti-Tac antibody was radiolabeled with tritium to high specific activity (910 Ci/mmol) by reductive methylation. Protein concentration was determined by three independent assays including quantitative amino acid analysis, colorimetric protein assay (Lowry/BioRad), and

absorbence spectroscopy. ^{3}H-anti-Tac was then employed in a radioreceptor binding assay to measure the number of IL-2 receptors expressed in varying cell populations. Binding with this ligand was specific, saturable, and reversible, thus permitting Scatchard analysis of the levels of bound and free ligand. In select experiments, anti-Tac binding was also evaluated by fluorescent activated cell sorting analysis (FACS). Binding assays to measure high and low affinity IL-2 receptors were performed with ^{3}H- or ^{125}I-labeled purified IL-2. Leukemic populations studied included: ATL, Sézary leukemia, acute T-cell leukemia, Burkitt's lymphoma, hairy cell leukemia, and pre-B-cell leukemia. Immunodeficient disease states studied included the acquired immunodeficiency syndrome (AIDS) and ataxia telangiectasia. Studies of IL-2 receptor expression in normal T cells have focused on the early rise and later decline in receptor number associated with T-cell activation and mechanisms for reinduction of receptor expression. Expression of IL-2 receptors on select subpopulations of normal activated B cells was also evaluated.

7) Evaluation of anti-Tac as a biologic modifier of the human immune response: Anti-Tac was analyzed for effects on antigen- and mitogen-induced T-cell proliferation as well as studied in assays of T-cell mediated cytotoxicity and B-cell immunoglobulin biosynthesis.

8) Anti-Tac-ricin A chain immunotoxins: Anti-Tac was covalently coupled to the A chain of ricin and studied for cytotoxic effects on HTLV-I-infected leukemia cells in vitro. Cytotoxic effects of this immunotoxin were monitored by measuring changes in leucine incorporation, directly assaying cell viability, and performing limiting dilution cell cloning assays. Effects of various lysosomotropic agents (e.g., NH_4Cl, chloroquine, and leupeptin) on immunotoxin killing were also studied. Finally, the capacity of these immunotoxins to kill tumor cells selectively but not inhibit stem-cell growth in human bone marrow was evaluated.

Major Findings:

IL-2 is a 15,500 dalton glycoprotein critical to the evolution of a normal human immune response. This ligand is not elaborated by resting T cells; however, following T-cell activation with antigen and interleukin-1 (lymphocyte activating factor), IL-2 is synthesized and secreted. IL-2 has been biochemically characterized, purified to homogeneity, its complete amino acid sequence determined, and post-translational processing events identified. Recently, the gene encoding IL-2 has been cloned and expressed in eukaryotic and prokaryotic cells. Like other polypeptide hormones, IL-2 acts through binding to high affinity specific membrane receptors. These receptors, however, are not present on resting T cells but like IL-2 are induced by exposure to antigen. Unlike IL-2, the IL-2 receptor has not been purified nor extensively characterized. We have identified a monoclonal antibody, anti-Tac, which recognizes the human IL-2 receptor. We have used this antibody to: biochemically characterize the IL-2 receptor; clone, sequence, and express cDNAs encoding this receptor; and modulate the human immune response in vitro.

Evidence that anti-Tac recognizes the human IL-2 receptor: We have established several lines of evidence indicating that anti-Tac is an anti-IL-2 receptor antibody. 1) This monoclonal binds to activated human T cells and IL-2-dependent T-cell lines but not to resting T cells, B cells, thymocytes, monocytes, or IL-2-independent T-cell lines. 2) Anti-Tac inhibits >95% of the binding of radiolabeled IL-2 to activated human T cells, and purified IL-2 blocks >90% of the binding of ^{3}H-anti-Tac to these cells. 3) Following DSS-mediated covalent crosslinking of purified IL-2 to radiolabeled HUT 102B2 cells, both anti-Tac and anti-IL-2 monoclonal antibodies immunoprecipitate a protein band 12,000-14,000 daltons larger than the free receptor. The protein size and immunoprecipitability by both anti-Tac and anti-IL-2 antibodies suggest that this new band is a receptor-ligand complex. This band is not identified in cells similarly treated but not covalently crosslinked with DSS. 4) Following ^{35}S-methionine or ^{3}H-glucosamine labeling of cells and extraction of membrane proteins in nonionic detergent, purified IL-2 coupled to affigel beads binds a protein identical in size to that recognized by anti-Tac bound to sepharose beads. Further, when these radiolabeled cell extracts are sequentially cleared with either IL-2 or anti-Tac affinity supports, subsequent precipitation with the other reagent fails to precipitate significant additional radioactivity. These data indicate that IL-2 and anti-Tac recognize the same membrane protein. 5) Anti-Tac but not anti-Ia antibody inhibits 70-85% of IL-2-induced proliferation in IL-2-dependent human T-cell lines. Taken together, these data strongly suggest that anti-Tac is an anti-IL-2 receptor antibody. Unlike IL-2, anti-Tac has no agonistic properties when added either alone or with a second crosslinking antibody. These data suggest that anti-Tac, while binding to the receptor and inhibiting IL-2 binding, does not react precisely with the same site as IL-2 or, alternatively, it does not produce the same conformational changes associated with IL-2 binding.

Biochemical characterization of the human IL-2 receptor: Following ^{35}S-methionine biosynthetic labeling of PHA-activated lymphoblasts, extraction in nonionic detergent, immunoprecipitation, and SDS-PAGE analysis, anti-Tac identified one major and two minor protein bands with M_rs of 55,000 (p55), 113,000 (p113), and 180,000 (p180). In contrast, anti-Tac immunoprecipitation of cells externally labeled with ^{125}I revealed only p55. These data indicate that neither p113 nor p180 display tyrosine residues on the external plasma membrane. p55 was also identified in cells labeled with ^{3}H-D-glucosamine, indicating that it is a glycoprotein. Further, IL-2 covalently coupled to affigel beads precipitated p55, indicating that p55 contains an IL-2 binding site. Analysis of p55 under nonreducing conditions revealed a migration with an M_r of 50,000, consistent with intrachain disulfide bonding. Isoelectric focusing of p55 indicated a pI of 5.4-5.7. To investigate whether p113 and/or p180 represented larger precursor forms of p55, pulse-chase labeling studies were performed. Both p113 and p180 labeled after p55 were identified, indicating that these proteins were not precursors of p55. However, in these studies, we identified two smaller proteins of M_r 35,000 and 37,000 (p35, p37) which labeled prior to p55 and disappeared as p55 was synthesized. These data suggested that p55 represented a processed form of these smaller precursors. To investigate this possibility further, cells were labeled with ^{35}S-methionine in the presence of tunicamycin which inhibits N-linked glycosylation. Following this treatment, p35 and p37 migrated as a

single band of M_r 33,000 (p33). Similarly, p55 was reduced in size by 2,000-4,000 daltons. Treatment of pulse-labeled cells with endoglycosidase F, which selectively digests N-linked sugars, recapitulated the findings obtained with tunicamycin. p35 and p37 migrated as p33 while p55 migrated as a slightly smaller protein. These data suggest that the IL-2 receptor is composed of 2,000-4,000 daltons of N-linked sugar attached to a protein backbone of 33,000 daltons. In pulse-chase labeling studies this precursor form was only detected in the presence of tunicamycin, consistent with cotranslational addition of N-linked sugars. Further studies of the large saltatory increase in apparent M_r from 37,000 to 55,000 daltons indicated that O-linked glycosylation and addition of sialic acid and sulfate were involved. Neuraminidase digestion of p55 revealed an apparent decrease in M_r of 6,000 daltons, but no change in the migration of p35 and p37. Sequential digestion with endoglycosidase F and neuraminidase provided evidence that sialic acid residues were present on non-N-linked carbohydrate structures, indirectly confirming the presence of O-linked sugar. IL-2 affigel beads also reacted with p33, p35, and p37, indicating that post-tranlational modifications were not obligately required for IL-2 binding. However, these data do not exclude the possibility that the affinity of the receptor for IL-2 is altered by post-translational processing.

Studies with the carboxylic ionophore monensin, which blocks Golgi-associated post-translational protein processing (including glycosylation), revealed blockade of the transition of p37 to p55. Interestingly, p35 was not detectable, suggesting that p35 is normally processed to p37 prior to further modification to p55. These data also indicated that the large saltatory increase in receptor molecular weight occurring 60-120 minutes after synthesis of the peptide backbone occurred in the Golgi apparatus.

Other potential post-translational modifications of the IL-2 receptor were also studied. PHA-activated lymphoblasts were incubated with ^{35}S-sulfuric acid to evaluate potential sulfation of the IL-2 receptor. SDS-PAGE analysis of immunoprecipitated cell extracts confirmed that the IL-2 receptor was sulfated; however, whether sulfate was introduced into carbohydrate (usually N-linked sugar) or into protein (occasionally at tyrosine residues) has not yet been determined. The radiolabel, however, was incorporated as sulfate rather than sulfide as indicated by complete precipitation of the radiolabel with barium chloride following acid hydrolysis of the labeled receptor. Like many other growth factor receptors, the human IL-2 receptor is also phosphorylated. Incubation of PHA-activated lymphoblasts with ^{32}P-orthophosphoric acid followed by solubilization in nonionic detergent and immunoprecipitation indicated that p55 was a phosphoprotein. However, addition of IL-2 did not appear to augment phosphorylation of this protein. These experiments, however, do not completely exclude an IL-2 inducible component of phosphorylation either involving p55 or other membrane proteins which may be part of a yet undefined receptor complex.

Molecular cloning, sequencing, and expression of the human IL-2 receptor cDNAs: The human IL-2 receptor was purified to homogeneity using an anti-Tac immunoaffinity support. The purified receptor, which retained IL-2 binding activity, was then sequenced by automated Edman degradation on a gas phase

microsequencer, permitting identification of the N-terminal 29 amino acids. Certain amino acid positions were confirmed by biosynthetic labeling, purification, and sequencing in a spinning cup sequenator. A synthetic oligonucleotide probe of length 17 and 64-fold degeneracy was then prepared based on protein sequence of amino acids 3-8 and used to screen a cDNA library prepared from HUT 102 mRNA in lambda gt10. The cDNA library was constructed using AMV reverse transcriptase in the presence of actinomycin D for first-strand synthesis, G-tailing, and oligo dC priming for second-strand synthesis with DNA Pol I, followed by EcoRl methylase protection and addition of EcoRl linkers, EcoRl digestion, and ligation into the EcoRl site of lambda gt10. The recombinant library contained 2.4 million phages with inserts ranging from 500 bp to several kilobases in size. This library was then amplified and 200,000 recombinant phages screened by Benton-Davis hybridization using the synthetic oligonucleotide probe kinased with ^{32}P. Eleven candidate clones for the IL-2 receptor were identified in serial hybridization screens, and each contained an insert which hybridized by Southern blot analysis to the 17 mer. Inserts from three of these candidate clones (clone 2, 0.9 kb; clone 3, 2.3 kb; and clone 4, 1.6 kb) were subcloned into pBR322 and analyzed for the capacity to selectively hybridize HUT 102B2 mRNA which when translated in a wheat germ lysate system and immunoprecipitated would reveal the primary translation product for the receptor. Each candidate receptor clone did selectively hybridize to the appropriate mRNA, providing additional support that these were IL-2 receptor clones. Each candidate cDNA was then subcloned into M13 and completely sequenced by the dideoxy chain termination method of Sanger. Each clone was found to contain a single long open reading frame including a continuous region of 87 nucleotides which corresponded to the 29 amino acids determined by protein sequencing, thus confirming their relationship to the IL-2 receptor. Analysis of the sequence of clone 4 indicated that this cDNA lacked a region of 216 nucleotides encoding 72 amino acids present in clone 3. Further, this region was bounded by classical mRNA splicing sequences (AGGT), suggesting that clone 4 had been transcribed from an mRNA which had spliced this apparent internal segment. In contrast, clone 3 was derived from an "unspliced" form of mRNA. Thus, we had evidence for two different cDNAs which when translated would produce proteins differing by 72 amino acids. In order to establish which cDNA corresponded to the IL-2 receptor, each cDNA was subcloned into the eukaryotic expression vector pcEXV-1. This plasmid contains SV40 promoter and enhancer elements, polyadenylation signals, and an EcoRl cloning site for insertion of foreign cDNAs. Following determination of insert orientation, each construct was transfected into COS-1 cells and analyzed for expression of IL-2 receptors (measured by ^{3}H-IL-2 binding) 72 hours later. We observed that transfection clone 3 cDNA, which retained the 216 base sequence, resulted in expression of IL-2 receptors, while the spliced cDNA of clone 4 did not result in a protein product capable of binding IL-2. Identical results were obtained in binding studies with ^{3}H-anti-Tac.

Using the IL-2 receptor cDNA clones, we next analyzed Southern blots of genomic DNA restricted with EcoRl, BamHI, and Hind III. These studies suggested that the IL-2 receptor is encoded by a single gene but did not exclude a recent gene duplication event. We next probed Northern blots using RNA from both resting and activated T and B cells. In activated T cells, we detected two distinct mRNAs varying markedly in size (1,550 nucleotides, 3,500

nucleotides). Each of these mRNAs contained the internal 216 base segment as determined by probing with a small restriction fragment isolated from the splicable region. Thus, we assume that both mRNAs encode a functional protein. This has been confirmed by identification of the receptor primary translation product from the two different size mRNAs separated by methylmercuric hydroxide gel electrophoresis. Utilizing a single-stranded M13 clone complementary to mRNA bridging the 216 base splicable region in S1 nuclease protection assays, we have confirmed that the aberrantly spliced cDNA corresponds to mRNA present both in normal activated T cells and five different ATL cell lines. Further analysis of the sequence of the cDNAs revealed the presence of a polyadenylation signal sequence (AATAAA) which apparently was not utilized since no poly A tract was present near this signaling sequence. Probes prepared from DNA fragments located 3' to this AATAAA site hybridized only to the large form of the mRNA. Thus, we assume that the two subclasses of IL-2 receptor mRNA are generated by variable use of this proximal and as yet unidentified more distal polyadenylation signal sequence. Select Northern blotting studies suggested that the smaller class of IL-2 receptor mRNA was actually a doublet. Utilizing S1 nuclease protection, we have found that two receptor mRNAs differing in length by approximately 225 nucleotides were present within this region. The smaller mRNA utilizes the sequence ATTAAA for polyadenylation while the larger employs the classical AATAAA sequence.

IL-2 receptor mRNA was not detected in resting peripheral blood lymphocytes but was present in PHA- and PMA-activated T cells as well as in HTLV-infected T-cell lines. Combined stimulation with PHA and PMA augmented the amount of the 16s form of IL-2 receptor mRNA. PHA- and PMA-induced, but not uninduced, JURKAT and HSB-2 acute lymphocytic leukemic T cells contained IL-2 receptor mRNA. B-cell lines infected with HTLV also expressed IL-2 receptor mRNA as did hairy cell leukemic B cells.

Analysis of the deduced amino acid sequence of the IL-2 receptor indicated a hydrophobic stretch of 19 amino acids near the carboxy terminus. This region probably represents a transmembrane domain which is long enough to span the membrane in an alpha helical configuration. Potential O-linked carbohydrate addition sites exist 5' to this region (serine and threonine residues), while a positively charged 13 residue region composed of basic amino acids is present 3' to this putative transmembrane domain. This charge cluster presumably anchors the protein on the cytoplasmic side of the membrane. Analysis of the NH_2 terminus indicates a signal peptide of 21 amino acids. Following signal peptidase cleavage, the protein is composed of 251 amino acids. Two N-linked glycosylation sites are present as well as 13 cysteine residues which may participate in intramolecular disulfide bonding.

Structure of the IL-2 receptor gene: Sequence analysis of overlapping genomic phage clones isolated by cDNA hybridization revealed that the receptor was organized as eight exons spanning at least 25 kilobases of DNA. In situ hybridization studies localized the IL-2 receptor gene to the short arm of chromosome 10 (10p 14-15). The exons correlated well with the peptide domains predicted by hydrophobicity analysis. Exon 1 encodes the 5' untranslated region as well as the signal peptide. Exons 2 and 3 contain the two N-linked glycosylation sites. The amino acid sequence of exons 2 and 3 is homologous to the recognition

domain of human complement factor B. Exons 4 and 5 bear significant homology to exons 2 and 3, suggesting the occurrence of an internal gene duplication event. This finding also raises the possibility that if these exons encode the binding site, then a single receptor molecule might bind two molecules of IL-2. Exon 4 precisely corresponds to the 216 base pair region which may be removed by aberrant splicing. Exon 6 contains multiple potential O-linked glycosylation sites while 7 and 8 encode the transmembrane domain. Exon 8 also contains the short cytoplasmic tail and the 3' untranslated region. This region contains multiple repetitive alu sequences between the second and third polyadenylation sites. Comparison of the sequence of the IL-2 receptor with all known DNA and protein sequences revealed no signficant homology except as noted above.

Expression of IL-2 receptors in varying neoplastic and normal cell populations: To quantitate IL-2 receptor expression in various cell populations, anti-Tac was tritiated by reductive methylation and used in a sensitive radioreceptor binding assay. Scatchard analysis of PHA-activated lymphoblasts indicated that when maximally activated (day 3), these cells expressed 30,000 to 60,000 IL-2 receptors. Anti-Tac bound to these cells with an apparent K_d of 1-2 x 10^{-10} moles/liter. The binding of anti-Tac was completely inhibited in the presence of a 100-fold molar excess of purified IL-2. Following PHA activation of these cells, IL-2 receptor expression was detected within 6 to 10 hours. Production of receptors by these cells required RNA and protein synthesis but not DNA synthesis. Northern blotting analysis of RNA isolated from lymphocytes stimulated with PHA for varying periods of time revealed detectable IL-2 receptor mRNA within 4 to 8 hours after activation. At time periods as early as 1 hour, a larger, presumably nuclear precursor RNA species was detected. Nuclear transcription assays indicated that IL-2 receptor gene expression occurred rapidly after PHA addition peaking at 6 to 15 hours. Culture of PHA-activated lymphoblasts in media supplemented with IL-2 permitted the long-term culture of these cells; however, following approximately 10 to 14 days of incubation, the rate of cell proliferation declined despite addition of large quantities of IL-2. To study this phenomenon, we measured IL-2 receptor levels at varying times late in culture. We noted that the number of receptors displayed declined during culture, suggesting that the T-cell responsiveness may not only be controlled by the amount of IL-2 present but also by the number of IL-2 receptors displayed. This fall in IL-2 receptor protein was preceded by diminished IL-2 receptor mRNA levels and decreased IL-2 receptor gene transcription. Thus, the rise and fall in IL-2 receptor expression occurring during normal T lymphocyte activation is controlled at least in part at a transcriptional level.

We next examined whether senescent activated T cells which had lost >90% of their IL-2 receptors could be reactivated to express greater numbers of IL-2 receptors. We observed that addition of PHA resulted in an increase in receptor number that was associated with increased cellular proliferation. PHA-induced increases in IL-2 receptor expression required intact RNA and protein but not DNA synthesis. Further, PHA augmented levels of IL-2 receptor mRNA in these cells as well as increasing transcriptional rate of the IL-2 receptor gene. IL-2 receptor reexpression also occurred following addition of PMA, phospholipase C, and synthetic congeners of diacylglycerol. Each of these three agents activated protein kinase C. Similarly, these agents resulted

in augmented levels of IL-2 receptor mRNA. Thus, protein kinase C may play an important role in the regulation of IL-2 receptor expression. Finally, we observed that IL-2 itself was capable of upregulating IL-2 receptor expression. These effects of IL-2 were similarly mediated through augmented IL-2 receptor gene transcription. Synergism between PHA and IL-2 in the induction of IL-2 receptor expression was observed at both the mRNA and protein level. Addition of large quantities of IL-2 during peak receptor expression did not prevent the progressive decline, suggesting possible active repression receptor gene transcription. Further, the fall in IL-2 receptor number was not associated with a relative increase in the spliced form of receptor mRNA. Thus, augmented post-transcriptional splicing of exon 4 does not appear to be involved in the progressive decline in IL-2 receptor expression occurring late in the process of normal T-cell activation.

In addition to induction of receptor reexpression with PHA and PMA, we observed that incubation of senescent PHA lymphoblasts with 5-azacytidine resulted in augmented receptor display. This nucleotide analogue, when incorporated into DNA, blocks methylation of cytosine residues. Methylation of transcriptionally active genes has been associated with gene inactivation. Alternatively, 5-azacytidine may also alter the normal progression of cells through the cell cycle, thus producing apparent differentiation analogous to 5-hydroxyurea. At this juncture we are attempting to define which mechanism is involved in 5-azacytidine induction of IL-2 receptor expression.

Analysis of differences in IL-2 receptor affinity for IL-2: Recent studies with purified radiolabeled IL-2 have demonstrated that IL-2 receptors may exist in high and low affinity states on the membrane of activated T cells. These different affinity classes of receptors are not discriminated by ^{3}H-anti-Tac. Further, the sum of high and low affinity receptors measured with radiolabeled IL-2 approximates the number of receptors measured with radiolabeled anti-Tac. The high affinity receptors, in general, comprise only 5-10% of the IL-2 binding sites in activated T cells. Functional studies indicate that these high affinity receptors mediate the growth-promoting response to IL-2. The function of the numerous low affinity IL-2 receptors, as well as the molecular and biochemical features which distinguish these affinity classes of receptors, remains unresolved. Recent studies with purified ^{125}I-IL-2 have demonstrated that only the high affinity form of the IL-2 receptor is internalized by receptor-mediated endocytosis. High affinity receptor internalization was detected with both murine CTLL cells and HUT 102B2 cells. Stable expression of IL-2 receptor cDNA in murine L cells resulted in the display of exclusively low affinity IL-2 receptors. As expected, these L cells did not respond to IL-2 with augmented growth. The high affinity IL-2 receptor may be produced by the formation of a receptor complex or, alternatively, produced by a particular form of post-transcriptional processing not occurring in the transfected L cells. Alternatively, while unlikely, we have not completely excluded the possibility that the high and low affinity IL-2 receptors are encoded by different genes.

We have also investigated IL-2 receptor expression in varying human lymphoid malignancies. Recent studies have focused on a leukemia of mature T cells termed adult T-cell leukemia (ATL). This leukemia has been etiologically

associated with HTLV-I infection. Thus far, virtually every case of ATL studied has displayed receptors for IL-2 as have continuous T-cell lines established from these patients' lymphocytes. Using the ^{3}H-anti-Tac radioreceptor assay, we have also noted that these HTLV-infected leukemic cells characteristically express 5- to 10-fold more receptors/cell than do maximally activated PHA-activated lymphoblasts. Further, since the cell volume of these leukemic cells is only 13% greater than that of PHA lymphoblasts, the density of IL-2 receptors on these leukemic cells is correspondingly increased. These findings are recapitulated in normal cord blood lymphocyte lines transformed with HTLV-I. We have found that some, but not all, HTLV-I-infected cell lines display aberrantly sized IL-2 receptors. The receptor in HTLV-I-infected HUT 102B2 cells is approximately 5,000 daltons smaller than that present in PHA lymphoblasts. Using pulse-chase, tunicamycin, endoglycosidase F, and neuraminidase analyses, we have demonstrated that this difference in receptor size is related at least in part to diminished post-translational addition of sulfate and sialic acid. The protein backbone and N-glycosylated precursors of the IL-2 receptor are identical in size in PHA blasts and HUT 102B2 cells, thus indicating that the size difference is generated during late Golgi-associated processing.

Analysis of the deduced amino acid sequence of the ATL cDNA and normal IL-2 receptor gene revealed that both were identical. Further, Southern blotting studies provided no evidence for rearrangement, amplification, or translocation of the IL-2 receptor gene in the HTLV-I-infected ATL cells. Northern blotting studies demonstrated constitutive production of large amounts of IL-2 receptor mRNA which underwent appropriate post-transcriptional processing. Nuclear transcription assays revealed high level constitutive transcription of the IL-2 receptor gene in these leukemic cells. However, in contrast to normal T cells, addition of PHA and PMA to ATL cells resulted in selective inhibition of IL-2 receptor gene transcription. These same agents activated IL-2 receptor expression in normal T cells. Analysis of promoter structure in normal and ATL cells demonstrated that normal T cells utilize two distinct promoters located immediately 5' to exon 1. In contrast, a third, normally cryptic, promoter is also utilized in ATL cells. Thus far, the unique association of IL-2 receptor expression with HTLV-induced infection is unexplained. Though unproven, IL-2 receptors may be involved in the malignant growth of these cells.

We have also studied IL-2 receptor expression by varying acute lymphocytic leukemic T cells. Thus far, we have not identified any acute T-cell leukemia which constitutively displays these receptors. This was not an unexpected finding in view of the generally immature stage of differentiation of these cells. However, we have demonstrated that select acute lymphocytic leukemic T cells (JURKAT, HSB-2) can be induced to express IL-2 receptors by stimulation with phorbol esters. Expression of receptors in these induced cells involves new mRNA and protein synthesis as indicated by studies with actinomycin D and cycloheximide. Of interest, the JURKAT IL-2 receptor is 2,000-3,000 daltons smaller than the IL-2 receptor present on PHA lymphoblasts. Scatchard analyses of ^{3}H-anti-Tac binding to induced JURKAT leukemic cells indicated approximately 7,000 receptors/cell as compared with 30,000 to 60,000 receptors/cell for PHA-activated lymphoblasts.

When activated with combinations of PHA and PMA, JURKAT leukemic cells were also induced to synthesize and secrete IL-2. In contrast, when these cells were activated with PMA alone, IL-2 receptor expression occurred but IL-2 production did not. Thus, the signals required for IL-2 production and IL-2 receptor expression are different. IL-2 production appears to require two signals, while a single signal (PMA) is sufficient for IL-2 receptor display. Further, these data indicate that ligand synthesis and secretion is not obligately linked with receptor display in these cells. As with reexpression of receptors in PHA lymphoblasts, PMA may induce receptor expression in these leukemic cells through activation of protein kinase C.

Further, in studies with Dr. Thomas A. Waldmann, IL-2 receptor expression on normal activated B cells has been detected. Cloned, activated normal B-cell lines have been established with EBV which express IL-2 receptors and bind radiolabeled IL-2 with high affinity. Further, IL-2 produces upregulation of IL-2 receptor expression in one of these cloned B-cell lines as well as augmenting immunoglobulin production. These findings have prompted a general reassessment of the role of IL-2 in the normal growth and maturation of human B cells.

Use of anti-Tac to modulate the human immune response: We have evaluated the capacity of anti-Tac to inhibit a variety of in vitro immune reactions with human lymphocytes. Anti-Tac blocked proliferation of human T cells stimulated with soluble, autologous, and allogeneic antigens. Anti-Tac partially inhibited T-cell proliferation induced by mitogenic lectins, and the degree of inhibition was inversely correlated with the potency of the mitogenic stimulus. Anti-Tac inhibition of antigen-induced T-cell proliferation was reversed by the addition of purified IL-2. Anti-Tac also completely abrogated the maturation of cytotoxic effector T cells in allogeneic cell cocultures; however, once formed, the effector function of these cells was not inhibited by anti-Tac. Further, anti-Tac inhibited T-cell-dependent B-cell immunoglobulin (Ig) production. The site of anti-Tac action in this system has not yet been defined. Anti-Tac may inhibit helper T-cell function; however, a direct action on human B cells is not excluded since, as noted above, certain activated B cells express IL-2 receptors and, in some systems, IL-2 enhances Ig production. Anti-Tac does not inhibit proliferation of purified B cells activated with EBV.

Evaluation of anti-Tac-ricin A immunotoxins: Previous studies had demonstrated that the proliferation of IL-2-independent HTLV-infected T-cell lines was not inhibited by addition of anti-Tac. In an attempt to develop a reagent which would selectively inhibit the growth of these leukemic cells, we conjugated the A chain of ricin to anti-Tac. The A chain of this lectin is a potent inhibitor of protein synthesis which acts through binding to the 60s ribosomal binding site for elongation factor-2. Anti-Tac-ricin A conjugates, but not control UPC-10-ricin A conjugates, produced marked inhibition of the growth of HTLV-infected leukemic cells measured by incorporation of ^{3}H-leucine. Concentrations of immunotoxin were identified which produced essentially complete inhibition of protein synthesis in these leukemic cells, while at the same concentration, UPC-10-ricin A had little or no effect. The inhibitory effects of anti-Tac-ricin A were blocked with purified IL-2 or unmodified

anti-Tac antibody, indicating that ricin A was coupled to the antibody and acted through binding to the IL-2 receptor. The cytotoxic effects on anti-Tac-ricin A were also enhanced by addition of lysosomotropic agency (e.g., NH_4Cl and monensin) which act by blocking intralysosomal degradation and increasing intracytoplasmic delivery of the immunotoxin. The cytotoxic effects of anti-Tac-ricin A were also confirmed in a limiting dilution assay. This assay system indicated that the immunotoxin was capable of killing 99.99% of the HUT 102B2 cells without overt nonspecific toxicity. Further, this immunotoxin, while effectively eliminating tumor cells seeded into fresh bone marrow samples, did not markedly inhibit the growth of bone marrow stem cells, thus raising the possibility of potential use of this reagent to purge tumor cells from autologous bone marrow prior to reinfusion.

Significance to Biomedical Research and the Program of the Institute:

The isolation of cDNAs and the gene encoding the human IL-2 receptor has permitted complete determination of the primary structure of this protein. This receptor plays a critical role in the regulation of the T- and perhaps B-cell immune response. Thus, these data will be useful in future studies aimed at defining the IL-2 binding site and elucidating the mechanism of membrane signal transduction. Further, expression of this gene in prokaryotic or eukaryotic vector systems may permit isolation of large quantities of protein which then may be used to develop reagents with agonistic or antagonistic properties. With these reagents, the capacity to regulate T-cell immune responses may become feasible. Finally, since the IL-2 receptor gene is an inducible gene product, it should provide a model system to further study gene activation in lymphocytes and define the tissue-specific and nonspecific factors which control this process.

The availability of monoclonal antibodies to the human IL-2 receptor may permit therapeutic manipulations of the immune response in various pathologic states including autoimmunity, graft versus host disease, and acute or chronic transplant rejection. Further, anti-Tac has already been employed diagnostically to distinguish HTLV-I-induced ATL from other clinically similar leukemias of mature T cells. Anti-Tac, either unmodified or complexed with select toxins or radionucleotides, may provide an alternative therapy for certain lymphoid neoplasms including ATL.

The availability of molecular probes to the IL-2 receptor will also permit further study of the intriguing, but presently unexplained, relationship of HTLV-I retroviral infection to high-level IL-2 receptor expression. The deregulated expression of IL-2 receptors in ATL may be critically involved in the malignant growth of these leukemic T cells.

Proposed Course of Research:

1) Studies to define the molecular and biochemical differences which distinguish high and low affinity forms of the human IL-2 receptor. As noted above, high (K_d of 10^{-11} M) and low (K_d of 10^{-9} M) affinity IL-2 receptors are displayed on the surface of activated T cells, usually at a ratio of approximately 1:10. The less numerous high affinity receptors mediate the growth-promoting

response to IL-2, while the function of the more numerous low affinity receptors remains unresolved. It seems likely that the production of the high affinity IL-2 receptor involves either the formation of a receptor complex or alternatively is produced by specific post-translational processing. At present, we are most attracted to the receptor complex model which, in view of the short intracytoplasmic domain of the IL-2 binding protein, might more readily explain the mechanism by which signal transduction through this receptor occurs. We shall use crosslinking studies with ^{125}I-IL-2 bound under high affinity conditions to attempt to define the possible presence of other proteins involved in the formation of high affinity receptors. Similarly, different detergents will be examined in immunoprecipitation experiments attempting to maintain the integrity of the putative high affinity receptor complex. Should other proteins be identified, monoclonal antibodies will be prepared and the relevant proteins purified as a first step toward the isolation of the corresponding cDNAs. Should such cDNAs be isolated, L cells expressing only low affinity IL-2 receptors will be cotransfected in an attempt to reconstitute high affinity IL-2 receptor expression. Retroviral vectors containing IL-2 receptor cDNA will also be prepared and expressed in human T cells to test for high affinity IL-2 receptor production.

2) Studies of the association of HTLV-I retroviral infection and IL-2 receptor display. Infection of human T or B cells with HTLV-I results in IL-2 receptor expression while infection of rat T cells with HTLV-I results in rat IL-2 receptor expression. We shall attempt to further study the relationship of this virus and IL-2 receptors focusing on the potential role of the LOR protein encoded by sequences within the pX region of HTLV-I. Haseltine, Rosen, and Sodroski have demonstrated that the LOR protein produces trans-acting transcriptional activation of the HTLV-I LTR. We shall study whether similar trans-activation of the IL-2 receptor gene as well as other cellular genes occurs following the introduction of LOR expression vectors into varying cell types. Similarly, using the IL-2 receptor promoter linked to the chloramphenicol acetyl-transferase gene, we will monitor whether the LOR protein is capable of enhancing the production of this indicator gene transcriptionally regulated by the IL-2 receptor promoter.

3) Production of chimeric receptors. We have noted that only the high affinity form of the IL-2 receptor is internalized by receptor-mediated endocytosis. Murine L cells stably transfected with IL-2 receptor cDNA do not internalize radiolabeled IL-2 since these cells exclusively display low affinity IL-2 receptors. The intracytoplasmic domain of receptors is believed to play an important role in this internalization process. We plan to exchange the intracytoplasmic domains of the low-density lipoprotein and IL-2 receptors. The LDL receptor undergoes rapid receptor-mediated endocytosis; thus, we shall test whether the resultant IL-2 receptor chimera containing the LDL receptor intracytoplasmic domain is capable of receptor-mediated endocytosis. Similarly, we shall test whether the LDL receptor with an IL-2 receptor intracytoplasmic domain loses the capacity to be internalized. We also plan to introduce the intracellular domain of the epidermal growth factor onto the human IL-2 receptor. We wish to evaluate whether the tyrosine-specific protein kinase encoded by this EGF receptor domain is activated by IL-2 binding. Similar chimeric receptor constructs will be prepared utilizing the intracytoplasmic domain of the human insulin receptor.

4) Studies to define the IL-2 binding site within the IL-2 receptor. Based on the deduced amino acid sequence, synthetic peptides and antibodies to these peptides have been prepared encompassing different regions of the IL-2 receptor protein. These reagents will be studied for their capacity to block radiolabeled IL-2 binding to its receptor. As well, site-specific mutations within the IL-2 receptor will be made and the mutant receptor expressed and studied for the capacity to bind IL-2. We are particularly interested in evaluating the potential role of the two N-glycosylation sites in modifying receptor expression and affinity for ligand.

5) X-ray crystallographic studies of the IL-2 receptor. We wish to attempt to crystallize the purified IL-2 receptor protein both in the presence and absence of purified IL-2 in order to define its tertiary structure with and without ligand. Large quantities of receptor protein are required for these studies. Thus, we have produced a secreted form of the IL-2 receptor by introduction of a translation termination codon immediately before the transmembrane domain of the receptor domain. Expression of this truncated IL-2 receptor cDNA results exclusively in the secretion of IL-2 receptor protein which will thus facilitate purification of large quantities of the receptor protein.

6) Mechanism of IL-2:IL-2 receptor signal transduction. We wish to investigate potentially important second "messengers" generated by the interaction of IL-2 with the high affinity IL-2 receptor. We shall investigate the potential role for activation of the phosphatidylinositol turnover, mobilization of intracellular calcium and import of extracellular calcium, activation of protein kinase C, and IL-2-induced phosphorylation of cellular proteins. We are also interested in investigating the presence and potential physiological role of nuclear receptors for IL-2.

Publications:

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SUMMARY REPORT
IMMUNOLOGY BRANCH
October 1984 - September 1985

The Immunology Branch carries out a wide range of laboratory investigations in basic immunobiology. Areas of particular emphasis include: 1) Regulation and control of immune responses; 2) Structure and function of cell surface molecules; 3) Immune effector cell mechanisms; 4) Transplantation biology; 5) Molecular biology and 6) Tumor immunology. In addition, the Immunology Branch maintains a fluorescence activated cell sorter facility which is involved integrally in many of the studies carried out in the Branch in each of the above areas, and is also used in a large number of collaborative investigations with other laboratories at NIH and elsewhere. This report summarizes research efforts in each of these areas during the past year. More detailed information on specific accomplishments can be found in the individual annual reports, as indicated numerically in the test below.

1. REGULATION AND CONTROL OF IMMUNE RESPONSES

Studies in Dr. Richard Hodes' laboratory have been extended to further analyze the function of antigen-specific and MHC restricted regulatory cells in B cell activation. It was demonstrated that similar regulatory mechanisms control the activation of B cell by antigen-specific and MHC restricted T helper cells or by autoreactive T helper cells. In addition the regulation of B cell idiotype expression was characterized in the T15-dominant response to phosphocholine employing cloned regulatory T cell populations (5108).

Studies of the alloreactive T cell repertoire were carried out in Dr. Hodes' laboratory employing large panels of cloned T cells. It was demonstrated that non-MHC as well as MHC encoded target determinants are recognized at high frequency by the population of autoreactive and antigen-specific T cells. Cross-reactive recognition of Mls^a encoded determinants was observed at an extraordinarily high frequency and was demonstrated to be MHC restricted. These unanticipated findings suggest a critical role for certain non-MHC determinants in the alloreactive T cell repertoire (5106).

Dr. Gene Shearer's laboratory has further extended studies of the immune deficiency state which is induced by chronic graft versus host reactions (GVHR). The observed deficiencies in ability to generate cytotoxic T lymphocytes (CTL) is associated with loss of IL2 production as well as loss of expression of IL2 receptors and helper T cell function. GVHR induced by combined class I and class II MHC allorecognition abrogated help for both self + X and allogeneic responses, whereas class II only allorecognition abrogated only responses to self + X, and GVHR induced by class I only MHC differences induced no immune suppression unless cytomegalovirus infection was concomitantly present. These studies are highly relevant to current clinical problems of GVHR induced in circumstances of bone marrow transplantation (5088, 5099).

Dr. Shearer has also extended studies of immune response suppression to the investigation of humans at risk for acquired immunodeficiency syndrome (AIDS). Subjects who are at high risk for AIDS or who had early symptoms of this syndrome were found to have decreased in vitro CLT responses to self + X antigens, but elevated T cell responses to HLA alloantigens. Peripheral blood

leukocytes (PBL) were demonstrated to be more efficiently infected with HTLV-III after activation with HLA alloantigens, indicating that cofactors such as susceptibility to HLA alloantigenic stimulation may contribute to the development of AIDS (5110).

Work from the laboratory of Dr. Alfred Singer demonstrated that two distinct classes of T helper cells participate in CTL responses against membrane bound antigens. $L3T4^+$ T helper cells responded to class II MHC antigens whereas $Lyt2^+$ T helper cells respond to class I MHC antigens. In contrast only the L3T4 T cell subset functioned as helper cells in responses to minor hiscompatibility antigens (5119). It was also demonstrated that T helper cells and T killer cells with identical Lyt phenotype and MHC restriction specificity nonetheless differed significantly in their antigen response repertoires, indicating potential differences in thymic selection of helper and cytolytic T cell populations. It was further demonstrated that class II restricted $L3T4^+$ CTL precursors expressed a class II restricted anti-TNP repertoire which was under stringent thymic influence, while in contrast the class II restricted $L3T4^+$ TNP specific helper T cell population was not under detectable thymic influence. Recent studies have also demonstrated that an $Lyt2^+$ inhibitory cell selectively blocks the generation of class II specific CTL, but not class I specific CTL, during in vitro sensitization culture (5111).

Dr. Howard Dickler's laboratory has extended its studies of immune regulation by idiotype networks through the generation and study of a monoclonal anti-idiotype antibody against anti-(T,G)-A--L antibodies. In vitro studies have demonstrated an influence of B cell genotype but not of regulatory T cells upon idiotype expression (5058).

Further family and population studies from Dr. Stephen Shaw's laboratory have indicated that DPw2 is a new marker for susceptibility to pauciarticular JRA, and that the disease gene marked by its association with DPw2 is distinct from those previously described in association with this disease (5100).

Dr. George Ting has demonstrated that the generation of CTL during in vitro culture requires differentiation factors in addition to IL2. These differentiation factors include a cytotoxic cell differentiation factor required for the activation of precursors into lymphokine induced cytotoxic cells, and a T cell differentiation factor required for the activation of antigen specific cytotoxic T cell precursors into CTL effectors (5118).

2. STRUCTURE AND FUNCTION OF CELL SURFACE MOLECULES

Dr. Howard Dickler's laboratory has produced a series of hybridoma antibodies which appear to recognize molecules selectively expressed on activated B lymphocytes. These molecules may be receptors for growth or differentiation factors (5035).

Studies from Dr. Richard Hodes have analyzed the determinants on Ia molecules recognized by alloreactive, autoreactive, and antigen-specific T cells. Cloned T cell populations responsive to given Ia molecules were differentially susceptible to inhibition by an extensive panel of monoclonal anti-Ia antibodies. These findings demonstrated the existence of multiple sites or conformations on an Ia molecule recognized by different T cells (5069).

Dr. Shaw and his colleagues have continued their investigation of the functional role of T cell surface markers in human cytotoxic T cell recognition. It was demonstrated that effector T cells and target cells form non-specific as well as specific conjugates, and that the specificity of cytotoxicity appears to function at the level of T cell activation rather than binding alone. In addition, the roles of accessory molecules in T cell recognition and activation have been evaluated. The T3 structure appears to be uniquely relevant to T cell triggering as demonstrated by the effect of immobilized anti T3 antibodies in T cell activation (5067). Cytotoxic T cells have also been used to define phenotypic differences between lymphoblastoid cell lines which are not distinguishable serologically, indicating the existence of previously unknown HLA encoded products or functions. Mutant cell lines are being generated and selected by CTL recognition in an attempt to further define T cell recognized determinants (5101).

The laboratory of Dr. Ronald Gress has studied the human anti mouse xenogeneic cytotoxic response in order to provide a model system for the study of human alloantigen repertoire, as well as to provide information relevant to xenogeneic transplantation studies. It was demonstrated that human CTL exhibit a fine specificity capable of distinguishing alpha 1 and alpha 2 domain changes in the class I MHC molecules of murine target cells. In addition, it was demonstrated that "accessory" molecules such as T3, T8, and the LFA determinants may express differential roles in allogeneic and xenogeneic recognition. Dr. Gress's laboratory has also generated a series of monoclonal antibodies specific for human T cell surface determinants. One appears to recognize an epitope of the sheep red blood cell receptor distinct from those recognized by previously characterized antibodies. A second antibody recognizes determinants expressed on all T cells and immunoprecipitates a T cell surface antigen of 90,000 molecular weight, consistent with specificity for the constant region of the human T cell receptor (5116).

Dr. David Segal and his colleagues have carried out studies with both T cell and ADCC effector cells showing that cross-linking of the receptors responsible for lysis to determinants on the target cell surface is sufficient to trigger lysis. Cross-linking of targets to other effector cell surface components does not lead to lysis. Thus, ADCC effector cells can be cross-linked to targets with heteroaggregates of anti-FcγR and anti-target cell antibodies. Similarly, cytotoxic human T cells can be targeted by anti T3-containing heteroaggregates. This approach appears to be useful in evaluating the functional role of T cell receptor molecules in cytotoxic effector function. In addition, it represents a potentially useful means for targeting effector cells against targets such as tumor cells in models of immunotherapy (5050).

3. IMMUNE EFFECTOR CELL MECHANISMS

The laboratory of Dr. Pierre Henkart has studied the molecular components of cytoplasmic granules from large granular lymphocytes (NK cells) and cytotoxic T lymphocytes. Studies have been carried out to determine the biochemical, immunological, and enzymatic properties of these granules. It was demonstrated that the cytolytic capacity of these lymphocytes appears attributable to a single potent protein cytolysin, which is capable of forming large membrane pores in the presence of calcium and lipid (5103, 5018).

4. TRANSPLANTATION BIOLOGY

Studies in the laboratory of Dr. David Sachs have been directed toward analysis of structure and function of MHC products, and in the manipulation of the immune response directed at these products. Additional strains of congeneic resistant recombinant lines have been developed and analyzed, and additional hybridoma cell lines generated (5021). Studies of mixed allogeneic and xenogeneic chimeras, in which radiated animals are reconstituted with mixtures of T cell depleted donor and host type marrow have been produced. It has been demonstrated that these animals are immunocompetent, and that tolerance is induced to donor type MHC determinants in these animals. This represents an important potential model for the clinical application of allogeneic and xenogeneic organ and tissue grafts in the absence of graft rejection responses by the host (5021).

In the past year, Dr. Bluestone's laboratory has continued to study the manipulation of transplantation responses with anti-receptor antibodies. This work has led to the production of antibodies directed at clonal receptor structures and other functionally related structures on T cells. These antibodies are being applied to in vivo and in vitro minipulation of alloreactive T cell responses (5112). Additional work from Dr. Bluestone's laboratory has centered on the fine specificity of anti H-2 specific CTL. These studies have shown that cytotoxic T cells generated across limited H-2 differences detect determinants distinct from those detected by monoclonal antibodies of similar fine specificity and can be used to define the structural determinants involved in MHC recognition by T cells (5117).

Dr. Sachs and colleagues have continued to use the inbred miniature swine model in transplantation biology studies (5023). Two recombinants within the MHC have been detected by progeny screening, and both recombinants involve separation of class I and class II encoding loci. The recombination has been confirmed at both the protein and DNA levels. Transplants of kidneys between recombinant haplotypes has indicated that selective matching for class II antigens permitted long term survival in 50% of animals tested, whereas skin grafts in the same combinations were promptly rejected. Following a kidney transplant, subsequent skin graft from the same donor showed prolonged survival, indicating that systemic tolerance had apparently been induced by the kidney graft. It therefore appears that class II tolerance may be particularly critical to allograft tolerance induction in experimental and perhaps clinical settings (5023). A series of monoclonal antibodies has been prepared against swine T cell antigens, and has been demonstrated to include antibodies which parallel OKT4 and OKT8, and are differentially expressed on porcine helper and cytotoxic T cells (5023). These antibodies are being further applied to studies of T cell elimination in bone marrow grafting models in the swine, where successful syngeneic reconstitutions of irradiated animals have been accomplished and where mixed allogeneic reconstitution experiments are in progress.

Studies from Dr. Alfred Singer's laboratory have examined the nature of the T cell subpopulations which function in in vivo graft rejection. These studies have suggested that the critical T cell subpopulation which initiates skin graft rejection is the IL2 producing T cell population, and that there exist two subsets of such cells which can be distinguished by both their Lyt phenotype and their MHC specificity (5122).

The laboratory of Dr. Ronald Gress has developed procedures for T cell depletion from human bone marrow by treatment with monoclonal antibody plus complement. These depletion procedures have been monitored by the use of a limiting dilution assay capable of detecting one residual T cell in one million marrow cells, and has been determined to produce residual T cell contamination in the range of one T cell per 10^4 to 10^6 bone marrow cells. This appears to be well below the 0.3% contamination level experimentally associated with GVH disease in mouse and man. Such T cell depleted marrows, characterized as to HLA type, extent of T cell depletion, viability, and stem cell progenator activity, are cryopreserved with the intent of application to clinical transplantation (5116).

A porcine class I MHC gene isolated in the laboratory of Dr. Dinah Singer has been introduced into the genome of the B10 mouse, where it is expressed on the surface of a variety of tissues. Skin grafts of such transgenic mice were rejected by normal B10 mice, suggesting that the foreign SLA antigen expressed on the mouse cell surface is recognized as a functional transplantation antigen (5124). Further studies will be directed at T cell recognition of these xenogeneic gene products both as foreign MHC determinants and as "self" restricting elements.

5. MOLECULAR BIOLOGY

The structure and genomic organization of class I MHC genes in the miniature swine have been examined by Dr. Dinah Singer and her colleagues. There are 6-8 class I genes, some of which encode classical transplantation antigens, whereas the function of others is unknown (5114). A novel class I MHC gene, which is only distantly related to the other members of the family, has recently been isolated and shown to be expressed in a variety of tissues (5123). The regulation of expression of the class I gene families has been examined and a variety of regulatory regions identified. Using a set of mutants, the positions of the transcriptional promotor and the interferon enhancer have been mapped (5115). The determination of the DNA sequences of two class I MHC genes in the miniature swine has established that the structure of class I molecules in the pig resembles those of other species. Furthermore, it is evident that the extensive polymorphism in this system is generated through variations confined primarily to the first and second protein domains of the molecule (5114).

Work in the laboratory of Dr. Kathleen Kelly has been directed toward the isolation and characterization of genes which are transcriptionally regulated following mitogen and lymphokine activation of lymphocytes. The c-myc oncogene has been shown to be transcriptionally induced as early as 1 hour after the activation of murine B cells with LPS or with murine T cells with Con A (5120). The murine c-myc and v-myb structural genes have been placed into constructs involving the murine metalothinein promotor, and these constructions have been introduced by transient gene transfer into cloned murine cytotoxic T cells. Initial results indicate that oncogene transfected cells generally show increased proliferative activity as assayed 48-72 hours after gene transfer. Currently the levels of mRNA resulting from the transcription of exogenously introduced DNA are being determined. In addition, subtractive cDNA libraries are being generated in order to isolate the set of genes activated by PHA stimulation of human peripheral blood T cells (5120).

6. TUMOR IMMUNOLOGY

Drs. George Ting and John Wunderlich have carried out studies of lymphokine induced cytotoxic cells (LICC). These in vitro induced cytotoxic effector cells were demonstrated to function in vitro and in vivo in preventing growth of both lymphoid and solid tumors (5118). Studies in the laboratory of Dr. John Wunderlich also demonstrated that antitumor cytotoxic cell responses of murine cells stimulated in vitro by IL2 are modulated in a strain dependent fashion by particular macromolecular polyanions. Several of these substances occur naturally in man and may serve as regulators of IL2 activity (5003).

The laboratory of Dr. David Segal has demonstrated that human peripheral blood lymphocytes can be targeted to kill human tumor cells using appropriate antibody heteroaggregates. These aggregates involve anti T3 antibodies coupled with antibodies that detect specific determinants on tumor cell surface. Further studies will investigate the potency of these targeted effectors both in vitro and in vivo as models of tumor immunotherapy (5050).

A controlled randomized clinical trial has been conducted comparing MeCCNU chemotherapy to immunotherapy with BCG or BCG plus allogeneic tumor cell vaccine for the adjuvant treatment of human malignant melanoma. A total of 181 patients was entered and treated, and preliminary evaluation reveals no significant differences between treatment groups in either recurrence or survival (5033).

7. FLUORESCENCE ACTIVATED CELL SORTER

The Immunology Branch Flow Cytometry Laboratory is staffed by Susan Sharrow and David Stephany and is under the supervision of Dr. Wunderlich (5062). Currently, 60 different projects are being supported, of which approximately 50% involve multiparameter analyses. The current workload is comprised of approximately 75% Immunology Branch projects and 25% projects from other laboratories at NIH and elsewhere. The facility daily performs 4-7 individual experiments consisting of a total of 150-250 samples. Currently supported projects include a broad range of applications, some of which are as follows: 1) quantitative measurement of cell:cell binding (conjugate formation) by dual laser FMF; 2) monitoring of the effects of recombinant IL2 therapy on human peripheral blood lymphocyte subsets (Drs. S. Lotze, S. Rosenberg, and colleagues); 3) characterization of specificity of monoclonal antibodies against MHC determinants and lymphocyte subsets in swine and man (5116, 5021, 5094, 5101); 4) the study of expression of a porcine class I MHC gene introduced into the genome of a B10 mouse, with the establishment of a transgenic line (5124); 5) the characterization of cell surface expressed MHC-linked gene products, including previously unappreciated products of the Qa-2 region; 6) cell surface phenotyping in order to detect low levels of specific cell types in bone marrow chimeras or in immunosuppressed animals (5023); and 7) extensive hybridoma screening (5021, 5023).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 05003-20 I

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cell-Mediated Cytotoxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, leboratory, and institute affiliation)*

PI:	J. R. Wunderlich	Senior Investigator	IB, NCI
Others:	C. C. Ting	Medical Officer	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	0.9	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Broadly reactive antitumor cytotoxic cell responses, generated in vitro by mouse splenocytes cultured with the lymphokine IL2, are modulated in a strain-dependent fashion by addition of polyinosinic acid (Poly I) to the cultures. Although Poly I is a synthetic polynucleotide, macromolecular polyanionic carbohydrates, some of which occur naturally in man, have been identified which act like Poly I. Studies of mouse strains whose splenocytes generate only low levels of antitumor cytotoxicity with Poly I, show that suppressive cell-cell interactions contribute to the low responses.

Project Description

Objectives: This project has been directed at understanding and manipulating mechanisms of cell-mediated immune cytotoxicity. Long-range goals are: (1) to identify and characterize factors which influence induction, maturation and expression of cytotoxic cellular immune responses particularly against tumor cells; (2) to define changes in cytotoxic cellular immunity which occur in tumor bearing hosts; and (3) to find means of manipulating the cytotoxic cellular immune response for therapeutic purposes.

Methods Employed: Cell-mediated cytotoxicity has been measured in vitro by release of ^{51}Cr from labelled target cells incubated with lymphoid cells for 2-6 hours. Cell-mediated cytotoxicity against syngeneic tumor cells has also been tested in vivo in standard tumor neutralization (Winn) assays in which effector lymphoid cells and tumor target cells are mixed together in vitro at room temperature and within 5 minutes injected subcutaneously into adult hosts. Hosts, which are either normal or pretreated with cytoxan, are observed for tumor growth and survival. Immune and unprimed cells have been collected from spleen, marrow, lymph node, thymus and peripheral blood. Cells have been fractionated by 1 g velocity sedimentation, density gradient separation in Percoll, removal of cells which ingest iron particles, removal of cells which adhere to anti-Ig coated petri dishes and lysis of cells by monclonal antibodies and complement. Cell purity has been monitored by flow microfluorometry with monoclonal antibodies against well-defined, cell-surface differentiation antigens and by tests of biological function. Hybridoma cell lines producing monclonal antibodies have been obtained from the Salk Cell Distribution Center, L. Herzenberg (Stanford) and D. Sachs (Immunology Branch). In addition effector cells have been produced by in vitro sensitization of lymphoid cells using previously established Mishell and Dutton tissue culture conditions. Broadly reactive effector cells have been generated primarily by culturing cells from normal mouse spleen, thymus or fetal liver with polyinosinic acid and/or growth factors in medium supplemented with syngeneic plasma. Mouse-derived growth factors have been generated by stimulating EL4 (C57BL leukemia) cells with a phorbol ester. Human-derived growth factor has been used in the form of highly purified recombinant interleukin-2 (IL2) produced in E. coli. and obtained from the Cetus Corporation through the courtesy of Dr. S. Rosenberg, NCI. Target cell lines have been adapted to growth in medium supplemented with mouse plasma. This medium also has been used to support cytotoxicity assays. Target cells have been provided by freshly explanted cells (including primary MCA induced tumor cells dispersed with highly purified collagenase and DNAse), tissue culture lines and established tumor cell lines passed in vivo or in vitro.

Sarcomas have been induced with methylcholanthrene (MCA) in mice which are routinely screened for potentially immunsuppressive pathogens before admission to the colony at the Frederick Cancer Research Center. Tumor cell lines established from these sarcomas are also screened for potentially immunosuppressive pathogens and contaminating virus-related antigens: mycoplasma (culture and serology) and a variety of viruses (serology) including MVM, Sendai, LDH and LCM. The tumor cell lines used in the study have been induced by a relatively low dose of MCA (100ug) and do not stimulate generation

of anti-tumor cytotoxic cells under standard in vitro conditions for generating cytotoxic cells against allogeneic transplantation antigens.

Major Findings: Previous work in this laboratory has demonstrated that normal mouse spleen cells cultured for 5 days in medium supplemented with syngeneic plasma and polyinosinic acid (Poly I) generate Thy 1^+ cytotoxic cells, whose broad pattern of target cell reactivity in ^{51}Cr-release assays includes freshly dispersed syngeneic primary tumor cells induced by MCA but not freshly dispersed or mitogen-stimulated lymphocytes. Broadly reactive Thy 1^+ effector cells generated in vitro also prevent tumor growth in vivo in Winn assays. A close relationship of these effector cells to natural killer cells is apparent from a) their development from unprimed lymphoid cells in the absence of stimulator cells, b) the broad pattern of target cell reactivity, defined by direct lysis and cold target cell inhibition tests, c) the phenotype of effector cells generated from splenocytes (Thy 1^+, NK 1.2^+, Lyt 1^-, 2^- - albeit asialo $GM1^-$, and d) the strain distribution pattern of high vs low responses. The observation that thymocytes generate this cytotoxic cell response in the presence of exogenously supplied growth factors, links the response at lease in part to the T cell lineage. The response is controlled by multiple genes, and there is evidence for gene dosage effects (F_1 responses intermediate between high and low parental responses and a unimodal distribution of backcross responses intermediate between the F_1 and low parent responses). This work has been extended this year by the following 3 major findings.

1) Previous findings showed that highly purified recombinant human IL2, a lymphokine which supports T-cell proliferation, will stimulate generation of NK-like antitumor activity by mouse splenocytes in vitro. This year we have found (1) that the strain distribution pattern of response levels is more uniform for IL2 than for Poly I, and (2) that the strain distribution pattern of response levels generated by the combination of Poly I and IL2 is dominated by Poly I. Thus even if splenocytes from a given mouse strain generate a high cytotoxic cell response in the presence of IL2 alone, the response is low with the addition of Poly I, if the response of the strain to Poly I alone is low. We speculate that in vivo IL2 alone is capable of inducing high levels of NK-like cytotoxic cell activity in most mouse strains, but that in some mouse strains a Poly I-like mechanism inhibits this response.

2) Poly I is not a naturally occurring substance. To determine whether naturally occurring substances act like Poly I in regulating the generation of NK-like activity, we have tested a variety of high molecular weight polyanions, which represent one of the mechanisms by which Poly I might operate. Stimulation of NK-like activity has been found in vitro with dextran sulfate, fucoidan, carrageenan, chondroitin sulfate, and glycophorin. The latter two substances are of particular interest because they occur naturally in animals, including man. The strain distribution pattern of response levels to dextran sulfate, the only new pattern tested so far, matches that found for Poly I. These observations suggest that there indeed exist naturally occurring substances, which modulate the NK-like response to IL2 by a Poly I related mechanism.

3) During the last year we have explored the mechanism by which Poly I induces high levels of NK-like activity in splenocytes from some mouse strains, but low levels in those from other strains. Splenocytes have been mixed from high and low responding strains which are H-2 matched but differ in expression of theta - a differentiation antigen expressed on Poly I-induced, NK-like effector cells. Phenotyping the effector cells generated by the mixed cells shows that splenocytes from high responding strains generate low responses in the mixed cultures. These findings suggest that suppressor mechanisms are associated with low level responses to Poly I.

Significance to Biomedical Research and the Program of the Institute: In contrast to other forms of lymphocyte-mediated antitumor activity, NK-like cytotoxic cells have shown considerable promise for tumor immunotherapy. This conclusion is based on findings with animal tumor models in a variety of laboratories, including ours, and on preliminary results of phase I clinical trials. Little is known, however, about the factors which regulate generation and function of NK-like cytotoxic cells. The issue of regulation of NK-like cells can be addressed directly using current in vitro techniques for generating NK-like cytotoxicity. Information from these studies will provide not only a more sound basis for immunotherapy with these antitumor cells but also will bear on the cellular basis for bone marrow graft rejection, nonspecific suppression of immune responses, and host defenses against viruses and parasites for which NK cell activity has been implicated.

Proposed Course of Project: We will continue to pursue the basis for mouse strain-related high vs. low cytotoxic antitumor responses by: 1) using target cell binding assays in collaboration with Dr. David Segal (Immunology Branch, NCI), as opposed to cytotoxicity assays, in order to distinguish between variability in the effector cell cytotoxic mechanism and variability in the generation of lymphocytes which bind to target cells; 2) testing for differences in the frequency of precursor cells by using limiting dilution assays; and 3) testing for differences in ancillary cells, which either help or suppress the response, by cell mixing experiments.

In addition, we will pursue the observation that naturally occurring substances have Poly I-like activity in regulating antitumor cytotoxic cell generation. Specifically we will determine if these substances 1) act in vivo, and 2) abrogate IL2-induced antitumor cytotoxicity in a strain-dependent fashion. In collaboration with NCI Surgery Branch staff, we will extend these observations to clinical cancer situations to determine 1) if Poly I-like substances block IL2-induced cytotoxic cell (LAK) responses in vitro for some but not all patients and 2) how blocked responses correlate with poor clinical responses to therapy with IL2 and LAK cells.

Publications:

Ting, C. C., Wunderlich, J. R., Hargrove, M. E., and Winkler, D.: In vitro and in vivo activity of lymphokine-induced cytotoxic cells. Int. J. Cancer, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05018-15 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Membrane Damage by Immune Mechanisms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. A. Henkart	Senior Investigator	IB, NCI
Others:	C. Yue	Medical Staff Fellow	IB, NCI
	W. Munger	Investigator	IB, NCI
	Y. Jiang	Visiting Fellow	IB, NCI
	T. Soares	Microbiologist	IB, NCI
	C. W. Reynolds	Investigator	BTB, FCRF, NCI
	H. Young	Expert	BTB, FCRF, NCI

COOPERATING UNITS *(if any)*

Arnold Greenberg, Manitoba Institute of Cell Biology, Univ of Manitoba, Winnipeg
Ruth Angeletti, Division of Neuropathology, Univ of PA School of Medicine

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The cytoplasmic granules purified from rat large granular lymphocyte tumors with NK activity have been studied to determine their role in cytotoxic and other functions of these lymphocytes. Biochemical purification of the cytolysin from the granules shows that it is a single peptide chain of about 65 kd. When erythrocytes are lysed with cytolysin, this molecule forms a large pore-like complex in the membrane which has been purified. Cytolysin activity was shown to be blocked by a variety of lipid analogues, in aggrement with a membrane insertion mechanism. Anti-idiotype antibodies raised against the phosphoryl-choline binding myeloma protein TEPC 15 were shown to bind to purified cytolysin and block its lytic activity, suggesting a choline recognition by cytolysin. The biological activities of non-cytolysin components of the granules were also studied. It was shown that a DNAse activity is associated with LGL granules in the Percoll gradient. This activity was not part of the cytolysin. The DNAse activity has a neutral pH optimum and is found in much lesser amounts in non-cytotoxic lymphocytes. Collaborative studies with other labs have led to identification of other granule components, including a factor which is chemotactic to large granular lymphocytes and other cells, and chromogranin, an antigen previously known only in neuro-endocrine cell granules.

Project Description

Objectives: Our overall objective is to define the mechanisms by which lymphocytes destroy foreign cells. To this end we have dissected the killing process into discrete steps which can be studied independently. In this project we have concentrated on the membrane lesion induced by lymphocytes in the target cell. Over the past few years, we have produced evidence that killer lymphocytes operate by a granule exacytosis mechanism and that one component in these granules is a cytolysin which inserts in the target cell membrane, compromising its permeability properties. Our current objectives are to understand this process at the molecular level and to define the other biologically active molecules in granules of large granular lymphocytes.

Methods Employed: In vivo passaged rat NK tumor cells were harvested from the spleens of leukemic rats, and purified by Ficoll Hypague. These contained $1-4x10^9$ cells, of which 80-90% are large granular lymphocytes. Cells at 10^8/ml were lysed by nitrogen decompression, the nuclei removed by Nucleopore filtration and 5 ml of the homogerate layered on 20 ml of 48% Percoll. Pure granules were harvested from the bottom region of the gradient. Cytolysin activity was measured routinely by serially diluting the material in PBS and adding an equal volume of SRBC suspended in BSS. Hemolysis was measured by hemoglobin release. DNAse was measured by release of ^{125} UDR from nulei.

Major Findings: The cytoplasmic granules purified from rat large granular lymphocyte tumors with NK activity have been studied to determine their role in cytotoxic and other functions of these lymphocytes. Biochemical purification of the cytolysin to greater than 95% has been accomplished by use of a heparin affinity column subsequent to the gel filtration step. The purified cytolysin is a single peptide chain of about 65 kd (which is one of the major granule proteins) which shows substantial change in mobility on SDS gels upon reduction. Confirmation of this molecule as the cytolytically active species comes from experiments in which membranes were prepared from erythrocytes lysed with cytolysin. When solubilized with deoxycholate and run on a sucrose gradient, a rapidly sedimenting complex was isolated which had the EM appearance of cylindrical pore-like complexes and a single protein band on SDS gels which reacted with anti-granule antibodies on Western blots. Cytolysin activity was shown to be blocked by a variety of lipid analogues, especially those containing choline. Experiments with anti-idiotype antibodies raised against the phosphoryl-choline binding myeloma protein TEPC 15 have shown both rabbit and mouse monoclonal anti-idiotype antibodies bind to purified cytolysin and block its lytic activity, suggesting a choline recognition by cytolysin. The biological activities of non-cytolysin components of the granules were also studied. It was shown that a DNAse activity is associated with LGL granules in the Percoll gradient. This activity was found in two fractions on the gel filtration column migrating at about 30 kd and 60 kd. The latter separated from the cytolysin on heparin columns. The DNAse activity has a neutral pH optimum and is found in much lesser amounts in non-cytotoxic lymphocytes. Collaborative studies with other labs have led to identification of other granule components. Arnold Greenberg has identified a factor which is chemotactic to large granular lymphocytes and other cells. Ruth Angeletti has found chromogranin, an antigen previously known only in neuro-endocrine cell granules.

Significance to Biomedical Research and the Program of the Institute: The process of lymphocyte destruction of foreign cells may be one of the most important mechanisms for the immunological rejection of allografts and tumors in vivo. Studies such as ours, directed at the cellular and molecular nature of this process, allow a more complete understanding of the basic knowledge of the body's immunological defense system against foreign cells, including malignant cells.

Proposed Course of Project: Study of the purified cytolysin to determine its mechanism of interaction with membranes will be a priority in our lab. The effects of calcium on purified cytolysin will be studied. Limited proteolytic digestion studies will be performed to identify protein domains in the molecule. Collaboration with Dr. Howard Young has already made considerable progress in identifying clones of a cDNA library of LGL tumor cells which express antigen recognized by anti-cytolysin antibodies. Such clones will be sequenced to provide detailed structural analysis of the protein, which in conjunction with the above studies, should allow a detailed picture of cytolysin mechanism. Monoclonal antibodies have been produced by collaborators at NeoRex and should provide useful tools for further studies of NK differentiation as well as cytolysin mechanism. Collaborative studies with Dr. Rebecca Pruss (NIMH) will assess the possibility that neuroendocrine mediators are present in granules.

Publications:

Millard, P. J., Henkart, M. P., Reynolds, C. W., and Henkart, P. A.: 1984. Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. J. Immunol. 132:3197.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05021-14 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Antigens Determined by the Murine Major Histocompatibility Locus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. H. Sachs	Chief, Transplantation Biology Section	IB, NCI
Others:	J. A. Bluestone	Laboratory Leader	IB, NCI
	S. L. Epstein	Senior Staff Fellow	IB, NCI
	S. Chatterjee-Hasrouni	Visiting Fellow	IB, NCI
	N. Shinohara	Expert	IB, NCI
	S. T. Ildstad	Medical Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies are being directed toward understanding the major histocompatibility complex, the structure and function of the products of this complex, and manipulations of immune responses to these products. Current studies include: 1) Characterization of major histocompatibility antigens: Congenic resistant strains of mice are developed, maintained, and used in serologic and immunochemical analyses of the MHC products of the mouse; 2) Studies of monoclonal antibodies to H-2 and Ia antigens: Hybridoma cell lines are produced by fusion of immune mouse spleen cells with mouse myeloma cells. The monoclonal anti-H-2 and anti-Ia antibodies produced by these hybridomas are analyzed by serologic and immunochemical means and are used to further characterize the fine structure of the MHC; 3) Characterization of receptor sites for histocompatibility antigens: Anti-idiotypic antisera are produced against anti-H-2 and anti-Ia hybridoma antibodies, and the effects of these antisera on in vitro and in vivo parameters of histocompatibility are assessed; and 4) Mechanism of tolerance to H-2 and Ia antigens: The humoral and cellular responses of radiation bone marrow chimeras are examined, and the mechanism for maintenance of tolerance in these animals is studied. Mixed allogeneic and xenogeneic chimeras, in which irradiated animals are reconstituted with mixtures of T-cell depleted donor and host marrow, are produced and the mechanism of tolerance and of immune responsiveness in these animals is studied in vivo and in vitro.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives: 1) To produce antibodies against the products of defined regions of the major histocompatibility complex. 2) To characterize the reactions of these antibodies with the cell surface of lymphocytes, and to determine the nature of the cells bearing individual antigens. 3) To characterize the products with which these antibodies react by immunochemical means. 4) To attempt to produce anti-idiotypic antibodies against the receptors on these antibodies which detect cell surface histocompatibility antigens. 5) To induce tolerance to major histocompatibility antigens by mixed bone marrow reconstitution, and to characterize the mechanism of responsiveness and tolerance to MHC antigens in these animals.

Methods Employed: 1) Congenic resistant strains of mice differing only at their major histocompatibility loci have been reciprocally immunized in order to produce antibodies of known, restricted specificity. The antibodies have been characterized by assays of complement-mediated lymphocytotoxicity and by fluorescence microscopy using a fluoresceinated rabbit antimouse immunoglobulin as a developing agent.

2) Hybridoma cell lines are obtained by fusion of immune mouse splenic lymphocytes with mouse myeloma cells (SP2/0, P3U1, and NS1). The cells are mixed and exposed to polyethylene glycol, 30% for 8 minutes, and fused cells are then cultured in the presence of a selective medium (HAT) for 2 weeks in microtiter wells. Hybridoma cells secreting anti-MHC antibodies are detected by a complement-mediated cytotoxicity assay on individual microwell supernatants. Positive cultures are then sequentially cloned in vitro and in some cases passed in vivo to produce large amounts of ascites hybridoma antibodies.

3) Cell surface antigens reactive with anti-H-2 and anti-Ia antibodies and monoclonal antibodies are isolated and studied immunochemically. Cells are labeled in vitro with ^{3}H-leucine or with ^{125}I, solubilized in a nonionic detergent, purified by lentil lectin chromatography, and then mixed with the antibodies being analyzed. Complexes are precipitated with Staphylococcus aureus Cowan I strain which contains protein A on its surface, and the precipitated complexes are then dissociated in SDS and mercaptoethanol and analyzed by polyacrylamide gel electrophoresis.

4) Hybridoma antibodies against H-2 and Ia antigens are purified by affinity chromatography on protein A Sepharose columns and are then used to immunize heterologous or allogeneic animals. Pig, rabbit, goat and mouse antihybridoma reagents have been produced. These reagents are absorbed exhaustively on normal immunoglobulins or myeloma proteins in order to remove anti-isotype and antiallotype antibodies, and the putative anti-idiotypic antibodies are then absorbed and eluted from the relevant hybridoma antibody. These anti-idiotypes are then analyzed by hemagglutination and hemagglutination inhibition assays using cells to which the immunizing hybridoma, different hybridomas, or normal immunoglobulins have been coupled.

5) Radiation bone marrow chimeras are prepared by lethal irradiation of recipients and reconstitution with bone marrow from allogeneic donors exsanguinated before harvest of long bones. As these animals mature, in vitro assays

are performed in an attempt to determine the mechanisms for the tolerance observed.

6) Mixed allogeneic and xenogeneic chimeras are prepared by combining various proportions of syngeneic and either allogeneic or xenogeneic bone marrow for reconstitution. Levels of chimerism are determined subsequently by complement-mediated cytotoxicity on peripheral blood lymphocytes and/or by flow microfluorometry using the florescence activated cell sorter. Levels of tolerance are assessed by skin grafting at 8 weeks following reconstitution.

7) Mice are treated in vivo with purified anti-idiotype antibodies. The effect of such treatment on idiotype levels and anti-H-2 antibody activity in the serum of these animals is examined. In addition, spleens from these animals are examined for in vitro MLC and CML reactivity. Treated animals are also examined for in vivo reactivity to appropriate skin grafts, and humoral and cellular immunity following grafting are examined by complement-mediated cytotoxicity assays and by CML assays.

Major Findings: 1) Fusion of spleen cells from mice hyperimmunized against H-2 antigens and boosted 2-3 days before fusion has been found to give satisfactory results in the production of anti-H-2 hybridomas. About 70 stable hybridomas have so far been produced, most of which detect H-2 or Ia antigens of a variety of haplotypes. Panel testing has indicated that most of these antibodies react with public specificities of the H-2 and Ia antigens, while a few appear to detect private specificities. Numerous crossreactions have been detected using these monoclonal antibodies, which define a variety of new public H-2 and Ia specificities.

2) Analysis of anti-H-2 antisera and monoclonal antibodies by gel electrophoresis of labeled cell surface antigens has demonstrated two new H-2 products determined by loci within the D region of $H\text{-}2^q$, named H-2L amd H-2R. Several hybridoma antibodies reactive with H-2L, H-2R and at least one additional antigen have been produced. These antibodies are being used for structural studies of additional H-2 molecules.

3) Antibodies directed against the killer cell have been shown to block cell-mediated cytotoxicity against the H-2 products. The specificity of these antisera has been shown to be directed to a product of a gene closely linked or identical to the Ly-2 locus. Additional antibodies have been produced by immunizing rats and mice with cloned cytotoxic T cell lines. Attempts are in progress to produce hybridomas from such immunized mice in order to determine other cell surface antigens of importance to the T cell cytotoxic phenomenon.

4) Evidence for nonspecific suppressor cell generation in bone marrow chimeras has been obtained at times up to 6 weeks following grafting. At later dates no evidence for suppressor cells of either specific or nonspecific nature was obtained, supporting the hypothesis of clonal deletion rather than active suppression. Mixed allogeneic and xenogeneic chimeras have been produced by grafting irradiated mice with graded mixtures of T cell depleted bone marrow from appropriate sources. Such mice are being evaluated for tolerance by skin graft challenge and by in vitro techniques.

5) Anti-idiotypic antibodies reactive with six of the hybridoma anti-H-2 antibodies and with four of the hybridoma anti-Ia antibodies have been produced and have been shown to be specific by a variety of assays. Assessment of hybridoma antibodies and immune sera for the presence of these idiotypic specificities has indicated that at least one of the anti-H-2 and one of the anti-Ia idiotypes are prevelant in the corresponding normal immune responses.

6) Treatment of animals with anti-idiotypic antibodies has been found to induce appearance of idiotype in the serum of these animals. This has been true for all of the anti-H-2 and anti-Ia anti-idiotypes so far examined. In addition, a percentage of the induced idiotype has been shown to bear the same anti-H-2 or anti-Ia specificity as the original monoclonal antibody. These findings therefore represent the induction of anti-H-2 and anti-Ia antibody responses in the absence of exposure to the actual antigens.

7) Following treatment with xenogeneic anti-idiotype in the 11-4.1 system, the major idiotype produced is on antigen non-binding molecules (Id'). Levels of these idiotype-bearing molecules fall gradually, but can be boosted by a skin graft bearing the H-$2K^k$ antigens. Control skin grafts do not lead to stimulation of these Id' molecules. These findings provide evidence for network interactions in the production of antigen non-binding idiotope-bearing molecules.

8) Mice reconstituted with mixtures of syngeneic plus either allogeneic or xenogeneic bone marrow have been found to manifest specific hyporeactivity to donor type skin grafts at 8 weeks following reconstitution. In the case of mixed allogeneic reconstituted animals, both host and donor lymphoid elements are found in the peripheral circulation, while only host elements can be found in the mixed xenogeneic reconstituted animals.

9) Assessment of genetic specificity of transplantation tolerance in mixed allogeneic chimeras has indicated that bone marrow reconstitution tolerizes completely to MHC antigens, but incompletely to skin-specific minor antigens encoded outside of the MHC. Similar skin-specific antigens may explain partial tolerance in the mixed xenogeneic (mouse plus rat) chimeras as well.

10) The effect of selectively depleting only host or only donor T cells in the mixed marrow inoculum has been investigated. Elimination of T cells from the donor was found not to be required in order to produce competent, tolerant animals. However, failure to eliminate T cells from host marrow led always to rejection of donor and animals that were not tolerant.

Significance to Biomedical Research and the Program of the Institute: 1) The H-2 and Ia antigens are cell surface determinants which appear to be involved in physiologic cell-cell interactions in the immune response. Therefore, antisera against these antigens provide tools for dissecting the mechanism of these cell interactions and possibly for modifying responses.

2) The specificity of the receptor for histocompatibility antigens should reside in the variable portion of the heavy and light chains of the relevant antibody molecules. Thus, anti-idiotypic antibodies against such receptors might be expected to distinguish those cells capable of reacting against individual histocompatibility antigens. Such antibodies thus provide an approach to modification

of the immune response to cell surface antigens. Our findings on induction of idiotype by in vivo treatment with anti-idiotype indeed indicate that such modifications are possible.

3) Mixed bone marrow inocula appear to lead to long-term survival and full allogeneic tolerance in bone marrow irradiation chimeras. Such conditioning, if possible with immunosuppression less drastic than full body irradiation, could provide a specific means of tolerizing for allografts of any organ, or perhaps even xenografts.

Proposed Course of Project: 1) In order to maintain isogenicity of the background of our congenic lines, backcrosses of these lines to the reference congenic partner will be performed at least once every ten generations. Backcrosses of congenic lines will be examined for further recombination events within the H-2 and I regions. Recombinants will then be examined for fine structure analysis of the MHC and for the production of antisera against new H-2 and Ia specificities.

2) Anti-idiotypic antibodies directed against anti-H-2 and anti-Ia receptors from hybridomas and from cytotoxic T cell clones will continue to be produced and studied. These reagents will be examined for reactions with antibodies and T cells produced in conventional immunization schemes in order to determine the prevalence of the individual combining sites.

3) The effects of anti-H-2 and anti-Ia anti-idiotypic reagents on MLC and CML reactions will be examined.

4) Further fusions of immunized cells from a variety of different strain combinations will be performed in order to produce additional monoclonal hybridoma antibodies to a variety of H-2 and Ia specificities. The library thus obtained will be screened by serologic and immunochemical means in order to further characterize the products of the MHC. Attempts will be made to identify products of new MHC genes, the presence of which has been suggested by DNA hybridization techniques.

5) The effect of in vivo treatment with anti-idiotype antibodies will continue to be examined. The effects of such treatment on skin graft rejection and on humoral antibody production and CML reactivity will be studied. The possible sharing of idiotypes between alloantigen receptors and modified self receptors will also be examined. Because anti-monoclonal antibody anti-idiotypes have not been found to react with cytotoxic T cells, anti-idiotypes against T cell receptors will also be examined for effects on in vivo humoral antibody production and skin graft projection.

6) Bone marrow transplantation studies will be continued, assessing the effect of mixed allogeneic and xenogeneic donor inocula on long-term tolerance to allografts and xenografts. Additional strain combinations for mixed allogeneic and xenogeneic reconstitution will be examined, and specificity of hyporeactivity will thereafter be assessed. Also, the effect of selective depletion of T cells from host and/or donor marrow elements will be examined. Mechanisms of hyporeactivity will be examined by techniques similar to those utilized for complete allogeneic chimeras. Attempts to abrogate presensitization against MHC antigens by mixed allogeneic reconstitution will be carried out. Also, an assessment of which cells within the bone marrow inoculum are responsible for long-term

tolerance will be attempted by specific elimination of cell populations with antibodies plus complement. Finally, attempts to adoptively transfer tolerance in vivo will be made.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05023-14 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transplantation Antigens of Swine

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. H. Sachs	Chief, Transplantation Biology Section	IB, NCI
Others:	S. A. Rosenberg	Chief, Surgery Branch	SB, NCI
	M. D. Pescovitz	Medical Staff Fellow	IB, NCI
	L. R. Pennington	Medical Officer	IB, NCI
	F. Popitz	Visiting Fellow	IB, NCI
	D. S. Singer	Senior Investigator	IB, NCI
	K. Sakamoto	Visiting Fellow	IB, NCI

COOPERATING UNITS *(if any)*

NIH Animal Center, Poolesville, Maryland
Joan K. Lunney, Research Chemist, USDA Animal Parasitology Institute, Beltsville, Maryland

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A breeding program has been carried out starting with two miniature pigs from different sources and selecting offspring according to tissue typing procedures aimed at defining the major histocompatibility complex of this species. By this procedure three herds of miniature swine, each homozygous for a different set of histocompatibility antigens at the MHC have been developed. Current projects include: 1) Assessment of survival of organs and tissue transplants among and between members of these herds as a model for tissue typing and transplantation; 2) Purification and characterization of the major histocompatibility antigens of this species, and isolation and characterization of peptides from these antigens for sequence analyses and for assessment of immunologic reactivity; 3) Assessment of the immunologic parameters involved in tolerance to allografts in this species; 4) Detection and characterization of intra-MHC recombinants. Two intra-MHC recombinants have been obtained and bred to homozygosity. Kidney transplants utilizing these new recombinants have shown that selective matching for Class II antigens frequently permits long-term kidney graft survival across a Class I difference. An additional MHC recombinant has been obtained recently which appears to provide a new combination of Class I and Class II genes; 5) Bone marrow transplants in miniature swine. The effect of mixing autologous plus allogeneic marrow in the reconstituting inoculum are being examined. This modality is being assessed as a specific preparative regimen for allogeneic organ transplantation; and 6) Production and characterization of monoclonal antibodies reactive with subsets of pig lymphocytes. Antibodies corresponding to many of the OKT series in man have been identified (including T4, T8, and T11). The effects of these antibodies on in vitro and in vivo transplantation immunity are being assessed, and they are also being used to assess mechanism of tolerance.

Project Description

Objectives: To develop and maintain three strains of miniature swine, each homozygous for a different set of histocompatibility antigens at the major histocompatibility locus (MSLA). The animals are used for in vivo experiments in organ and tissue transplantation and as a source of large numbers of cells from which cell surface antigens are isolated, purified, and characterized chemically.

Methods Employed: Unlike classical inbreeding schemes which require about twenty generations to produce homozygosity, the approach used with these swine involved selective breeding on the basis of histocompatibility typing of parents and offspring.

Four males and four females were purchased from commercial sources, and mating pairs were selected to be as varied as possible in order to assure the selection of different histocompatibility genes. Skin grafts were exchanged between the members of each pair and sera were obtained from the animals two weeks after the rejection of the grafts. The sera were tested for cytotoxic antibodies by lymphocytotoxic typing, and pairs which produced strong cytotoxic antibodies were bred.

Offspring from each breeding were tested serologically to determine which histocompatibility antigens had been inherited from the parents, and offspring which could be shown to possess the same antigen combinations were selected for further breeding. This process has now been repeated for eight consecutive generations.

Transplantation of tissues in these animals has been performed in the large animal facility of the Surgery Branch in Building 14. Methods have been developed for the transplantation of split thickness skin grafts from the ear to the dorsal thorax. Allografts are always placed side by side with autografts as a control. The use of ear skin permits very accurate assessment of viability of the grafts and determination of rejection times. Surgical techniques for vascularized grafts (kidney and liver) have been developed.

In order to produce large quantities of histocompatibility antigens without the use of radiolabeled amino acids, membranes have been prepared from lymphoid organs and solubilized with detergents or by limited papain digestion. The extracts were then purified by lentil lectin chromatography and by affinity chromatography on anti- 2 microglobulin columns, or, in the case of papain solubilized antigen, by DEAE ion exchange chromatography and by gel filtration. Sera produced between outbred swine which react with public specificities shared in the inbred minipig population are being used to separate products of different histocompatibility loci. Amino acid sequencing of heavy and light chains from purified SLA antigens is being performed. Preparation, isolation, and characterization of peptides from these isolated antigens have been initiated.

All progeny of heterozygous animals have been screened by serology and by MLC reactivity to detect intra-MHC recombinants. Monoclonal antibodies against swine cell surface antigens have been prepared by immunizing mice with thymocytes from one of the partially inbred herds (SLA^{dd}), and then fusing the mouse spleen with SP2/0 to produce hybridoma cell lines. The cell lines have been screened for activity by cytotoxicity and fluorescence activated cell sorter analysis.

In collaboration with Dr. Dinah Singer, genomic clones encoding SLA antigens are isolated and characterized. Characterization includes detection of SLA products on transfected cell lines by antisera and cellular assays.

Animals are lethally irradiated with 1,000R from a bilateral cobalt source and are then reconstituted with either autologous (harvested the previous day), allogeneic or mixed autologous plus allogeneic bone marrow. Animals are observed closely and monitored for reconstitution of hematopoietic and lymphoid elements. Long-term survivors are examined in vitro and in vivo for effects on parameters of transplantation immunity, including skin and organ grafts, MLC and CML reactions, and antibody production.

Major Findings: 1) Breeding of further generations has continued to be successful. We therefore now have three different herds of swine, each homozygous for a different SLA antigen, as well as two herds of recombinant haplotypes.

2) Skin graft survival has been found to be prolonged within each of the homozygous herds. The mean survival time for grafts within homozygous herds was 11.8±.89 days, while the mean survival time of skin grafts between animals of the three different herds was 7.0±.36 days.

3) Renal allografts within the DD homozygous herd appeared to survive indefinitely despite minor histocompatibility antigen differences. Rejection occurred following allografts within the other two herds, with variable kidney survival times.

4) Skin grafts to DD recipients maintaining a DD allografted kidney showed marked prolongation of survival. DD kidneys transplanted after skin graft rejection by DD recipients were rejected in hyperacute fashion, but no antibody was detectable.

5) Alloantisera between the three herds have been analyzed by gel electrophoresis using detergent solubilized cell surface antigen preparations. Peaks were obtained at 45,000 molecular weight corresponding to the mouse H-2 antigen analog. Peaks at 35,000 and 28,000 molecular weights corresponding to mouse Ia antigens were also observed. Both by size criteria and by genetic criteria these antigens thus appear to be the precise homologs of H-2 and Ia antigens of the mouse.

6) N-terminal amino acid sequences have been obtained for SLA antigens isolated from all three of our partially inbred lines. Comparisons of these sequences with each other and with sequences of MHC antigens from other species reveal high levels of homology, as well as possible allotypic differences.

7) Two recombinants within the MHC were detected by screening of the progeny of MHC heterozygous animals using MLC reactivity and the cytotoxic assay as markers. Both new recombinants involved separation of the MLC stimulatory locus (SLA-D) from the serologic loci (SLD-A,B,C). SDS-PAGE analyses of cell surface antigens from these animals have indicated that the Ia antigens segregated with the MLC stimulating determinants in both recombination events, confirming the identity or close linkage of the genes responsible for both of these products in this species.

These recombinants have also been used to produce large amounts of antisera specific for Ia or SD antigens of this species, which were not previously available. An additional recombinant has recently been identified which separates the Class I genes of the SLA^a haplotype and the Class II genes of the SLA^d haplotype. The recombination has been confirmed at both the protein and DNA levels. Attempts are being made to breed this animal in order to establish this new recombinant haplotype.

8) Transplants of kidneys between recombinant and nonrecombinant haplotypes have permitted evaluation of the effects of selective Class I or Class II matching on renal allograft survival. In approximately 50% of animals tested, selective matching for Class II antigens permitted long-term survival. Skin grafts in the same combinations were rejected promptly, but following a kidney transplant a subsequent skin graft from the same donor showed prolonged survival, indicating that systemic tolerance had apparently been induced by the kidney graft.

9) A series of monoclonal antibodies reactive with a variety of swine lymphocyte surface antigens have been prepared. One of these antibodies recognizes an SLA antigen of the SLA^{dd} haplotype, at least two others react with selective T cell subpopulations, and at least one other antibody is reactive with macrophages. Further characterization of these antibody reactivities is in progress.

10) A genomic clone encoding SLA^{dd} antigen has been isolated and shown to cause expression of SLA^{dd} determinants on L cells following transfection.

Significance to Biomedical Research and the Program of the Institute: One of the major problems in the study of cell surface antigens of human beings is lack of control of genetic constitution. The use of mice and rats as experimental models avoids this problem, but creates two new ones: 1) physiologically and anatomically these animals are often so different from human beings as to make comparisons and applications of findings difficult. 2) The size of these rodents makes it extremely difficult to obtain sufficient cells and tissues to permit quantitative chemical characterization of relevant cell surface antigens.

For both of these reasons it is desirable to have animals of size comparable to human beings, whose genetic constitution with respect to histocompatibility can be controlled. The miniature pig, which attains an adult weight of about 200 lbs, is ideal for this purpose, and to date the experimental breeding pattern outlined above appears to be working well.

The availability of large quantities of MHC antigens make it possible to determine the effects of soluble antigen and possibly of peptides from these antigens on tissue transplantation. This will be assessed both by in vitro assays (MLC and CML), as well as in vivo in the transplantation models which have now been developed in these swine. In addition, large amounts of soluble SLA antigens should permit both primary and secondary structural studies to be performed.

The availability of monoclonal antibodies reacting with subsets of lymphocytes in the pig corresponding to similar subsets in man permits an analysis of the effects of such antibodies (e.g., elimination of T cell subsets) on parameters of transplantation immunity which should be directly applicable to human studies.

Proposed Course of Project: The breeding plan and typing will be continued. Attempts to identify new recombinants by serologic and MLC screening of progeny from heterozygous breeders will be continued. Recombinants thus obtained will be bred to homogeniety as has been accomplished for the first two recombinants.

Experiments in collaboration with the Surgery Branch will be continued in order to: 1) characterize the immune response to transplantation of skin, kidney, and liver in pigs across defined histocompatibility differences, and 2) to determine the basis of allograft tolerance which has previously been reported in pigs.

Studies of the MSLA antigens at the biochemical level will be continued. Using the isolated unlabeled histocompatibility antigens, we will begin to prepare peptide fragments of these antigens and to analyze these fragments for immunologic reactivities. An attempt will be made to localize those determinants involved in serologic assays (by inhibition of complement-mediated lysis), as well as in cellular assays (CML and MLC). The antigens will also be subjected to further sequence analysis in an attempt to determine the structural basis for antigenicity in this system. An attempt to crystallize the SLA antigens for x-ray crystallographic studies will be made in collaboration with Dr. David Davies (Laboratory of Molecular Biology, NIADDKD).

Investigations of the mechanism of tolerance induced by a vascular graft across a Class I only difference will be carried out. These will include assays both of cellular immunity (e.g., suppressor T cells) and of humoral immunity (IgM-IgG switch, soluble factors, etc.).

As a follow-up to our findings in the mouse model, bone marrow transplantation studies in miniature swine will be continued. The effects of mixing autologous plus allogeneic marrow on the outcome of bone marrow transplants will be examined. This methodology will be assessed for its effects on subsequent transplants and in vitro parameters of transplant immunity.

Because of the marked effect of Class II matching on renal allograft survival, an attempt to isolate and characterize the Class II genes of our pigs will be made at the DNA level. Attempts will also be made to transfect these genes into normal cell populations in order to obtain expression, and eventually in order to transfect into bone marrow for purposes of inducing transplant tolerance.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05033-14 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunotherapy of Human Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Hodes	Chief, Immunotherapy Section	IB, NCI
Others:	S. A. Rosenberg	Chief	SB, NCI
	R. I. Fisher	Senior Investigator	MB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

D

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A controlled, randomized trial comparing immunotherapy to chemotherapy in stage I and stage II malignant melanoma has been initiated. A total of 181 patients have entered the trial, which is closed to further accrual of patients. Preliminary evaluation of data has demonstrated no significant effect of adjuvant therapies on clinical course.

GPO 914-918

Project Description

Objectives: This project is designed to study various approaches to the immunotherapy of human malignancies.

Methods Employed: A new protocol (designed IB-2) was initiated in August 1975. This protocol evaluated the effect of two types of immunotherapy or one type of chemotherapy on the remission duration and survival of patients with stage I (level 4 or 5) and stage II malignant melanoma. Patients were stratified by stage, site of primary, and for stage II patients, presence or absence of clinically palpable lymph nodes and the number of histologically positive lymph nodes. Following stratification, they were randomized to receive no further therapy (this is standard treatment for these patients); or chemotherapy with methyl-CCNU; or immunotherapy with BCG; or immunotherapy with BCG plus a vaccine that consists of three tissue culture-grown allogeneic melanoma cell lines treated with the enzyme neuraminidase to remove sialic acid (and thus render them more immunogenic) and frozen until ready for use.

Following randomization, patients were assigned to the Medicine Branch for chemotherapy, the Immunology Branch for immunotherapy, and the Surgery Branch for follow up if there is no further treatment.

Bloods were drawn during pre-randomization work-up and throughout the treatment cycle for the purpose of serum banking.

Major Findings: As of Dec. 1, 1982, 181 patients had been randomized into this protocol. The state of the trial is summarized in the following table:

	Control	MeCCNU	BCG	BCG & Vaccine
Total Patients Entered	43	46	47	45
Recurrences	32	25	38	25
Deaths	24	23	27	21

In vitro assays have only been carried to the point of indicating that patients are being effectively immunized with the vaccine (see Project No. Z01-CB-05016-10 I). In addition, PPD tests indicate that all patients receiving BCG have converted to a positive skin test.

Significance to Biomedical Research and the Program of the Institute: Immunotherapy studies will explore the clinical effectiveness of manipulating the immune system in patients with cancer, and will also provide new information about the biology of the tumor-host relationship.

Proposed Course of Project: No further patient accrual will occur. All treatment has been completed and, for those patients already on study, follow-up as described by the IB-2 protocol will be continued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05035-13 I

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Function of B Lymphocyte Surface Membrane Molecules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. B. Dickler	Senior Investigator	IB, NCI
Others:	F. Uher	Postdoctoral Fellow	IB, NCI
	M. Principato	Postdoctoral Fellow	IB, NCI
	E. Park	Postdoctoral Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.75	3.75	1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to characterize the function of B lymphocyte membrane molecules. Previous findings indicate that the Fcγ receptors of B lymphocytes interact with: a) the lymphocyte cytoskeleton, b) Ia antigens, c) LyM antigens, d) surface IgM, and e) surface IgD. Each of these interactions is distinct, specific, and non-random. Studies with a B lymphocyte hybridoma (2.4G2) have shown that this hybridoma produces a low molecular weight substance(s) which triggers B lymphocytes to both proliferative and secrete antibody. This result suggests that certain B lymphocytes may produce factor(s) with helper activity. Studies utilizing antigen-antibody complexes and monoclonal anti-Fcγ receptor antibodies indicate that B lymphocyte Fcγ receptors cross-linked by their physiologic ligand down-regulate B lymphocyte differentiation without affecting proliferation. Resting but not activated B lymphocytes are susceptible to this negative regulation. Occupancy of B lymphocyte surface IgM by a separate ligand is necessary for inhibition to occur, suggesting that the previously described interaction between these two membrane receptors is involved in generating the negative signal. Monoclonal anti-Fcγ receptor antibodies on a Sepharose matrix but not in soluble form affect B lymphocyte function in a fashion similar to antigen antibody complexes. This result suggests that certain monoclonal antibodies specific for cell surface receptors must be presented in a form which will produce effective cross-linking in order to obtain functional effects. Covalently cross-linked monoclonal antibodies are being prepared to test this hypothesis. Recently, several monoclonal antibodies have been produced which react primarily with activated B lymphocytes. The molecules recognized by these antibodies will be characterized chemically and functionally. These molecules may be receptors for growth or differentiation factors.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: Characterization of the function of B lymphocyte membrane molecules including identification of receptors specific for lymphokines which stimulate or trigger B lymphocytes.

Methods Employed: For exaluation of interactions between B lymphocyte membrane molecules, ligands or antibodies (including monoclonal antibodies) specific for different membrane molecules are labelled with different fluorochromes, and then allowed to interact with lymphocytes. Binding and distribution are assessed both quantitatively and qualitatively using flow microfluorometry or fluorescent and phase microscopy. The interactions are studied by manipulation of the conditions of interaction, and by immunologic and chemical modifications of lymphocytes and/or ligands. For evaluation of the function of B lymphocyte membrane molecules, rigorously T lymphocyte-depleted spleen cells from mice are cultured with ligand (or monoclonal antibodies as model ligands). Cultures with ligand alone are assessed for stimulation of responses, while cultures containing both ligand and a) $F(ab')_2$ anti-mu and lymphokines, or b) the mitogen LPS (as stimulatory signals) are assessed for augmentation or inhibition of responses. The responses assessed are proliferation (^{3}H-thymidine incorporation) and antibody secretion (either by direct or reverse plaque forming cell assay or Elisa analysis of culture supernatants). Hybridomas which react primarily with molecules expressed on activated B lymphocytes are prepared by immunizing rats with activated murine B lymphocytes and subsequent fusion of the spleen cells from these rats with rat myeloma fusion partners by standard techniques. Resultant hybridomas are cloned by limiting dilution and screened for reactivity with various cell types by cell surface Elisa. Molecules of interest are further characterized by flow microfluorometry, immunochemical techniques, and functional analysis as described above.

Major Findings: A series of cell surface molecules (Ia antigens) is encoded by genes within the I region of the murine H-2 complex. These are either identical to or closely linked to genes (immune response genes) which regulate immune responsiveness. Anti-Ia antibodies bound to Ia antigens inhibit binding of immune complexes (heat-aggregated IgG or antigen-antibody complexes) to specific cell surface receptors (Fcγ receptors). The inhibition is specific: (a) the Fc portion of the anti-Ia antibody is not required; (b) binding of ligands to other cell surface molecules (including H-2K, H-2D, IgM, IgD, and lectin receptors) does not produce inhibition; (c) anti-Ia antibodies bound to Ia antigens do not inhibit detection of other surface molecules; and (d) inhibition is observed with some cell types (B lymphocytes and a subpopulation of T lymphocytes) but not others (macrophages and null lymphocytes).

The nature of the Ia antigen-Fcγ receptor interaction has been examined: (a) these molecules are not identical since binding of antibodies to only a portion of Ia antigens produces maximal inhibition of Fcγ receptors and the latter can be redistributed without affecting distribution of Ia antigens; (b) ligand-bound Ia antigens do not appear to bind directly to the same site on Fcγ receptors as immune complexes since occupancy of these receptors by Ig complexes but not ligand-Ia antigens results in

an interaction with surface IgM (see below); (c) the interaction does not appear to be mediated by the cytoskeleton since disruption of cytoskeletal function by drugs does not affect the interaction; and (d) the interaction may be steric since monoclonal antibodies against single antigenic determinants on Ia antigens produce only partial inhibition of Fcγ receptors whereas mixtures of the same monoclonal antibodies produce maximal inhibition; however, immune complexes bound to Fcγ receptors do not sterically inhibit detection of Ia antigens. Thus, while the nature of the Ia antigen-Fcγ receptor interaction has not been fully elucidated, the simplest interpretation is that the two molecules lie in close proximity on the cell surface. A functional role for the interaction is suggested by the non-random and specific nature of the association.

Binding of ligand ($F[ab']_2$ anti-Mu) to surface IgM of B lymphocytes or ligand-induced redistribution of surface IgM has no effect on Fcγ receptors. However, if the latter receptor is occupied by antigen-antibody complexes which themselves provide insufficient cross-linking to cause redistribution or monomeric IgG at physiologic concentrations then ligand-induced redistribution of IgM results in redistribution of the Fcγ receptors. The interaction is specific and unidirectional: (a) redistribution of Fcγ receptors by further cross-linking does not result in redistribution of monomeric ligand occupied IgM; (b) ligand-mediated redistribution of IgM does not result in redistribution of ligand occupied Ia antigens or monomeric ligand occupied IgD; (c) cross reactions between ligands was excluded. A smilar interaction has been demonstrated for surface IgD and Fcγ receptors except that the interaction only occurs on a subpopulation of B lymphocytes, and only with complexed IgG but not monomeric IgG. These results suggest that whenever IgM is involved in a B lymphocyte response, then the Fcγ receptor is also involved, and the differences between the sIgM-Fcγ receptor and sIgD-Fcγ receptor interactions provide a mechanism whereby the two antigen receptors could provide different signals to the B lymphocyte.

The hybridoma 2.4G2 but not the parental fusion partner P3U1 produces a low molecular weight factor which stimulates proliferation and antibody secretion from B lymphocytes in the absence of T lymphocytes. Responses to this factor are polyclonal and mainly of the IgM class. Responses were obtained primarily from larger B cells which bear the LyB5 alloantigen. Adherent accessory cells were not required for these responses. These results suggest that some B cells may produce factor(s) which promote growth and/or differentiation of B lymphocytes.

Experiments with antigen-antibody complexes (IgG anti-ovalbumin-ovalbumin or IgG anti-IgG-IgG) have revealed that such complexes inhibit the antibody secretion (60-70%) of normal B cells responding to $F(ab')_2$ anti-mu plus lymphokines. Complexes in antigen excess were most effective. Maximal inhibition was seen with as little as 0.2-0.5 μg/ml of complexes. Antibody or antigen alone were without effect and no effect on proliferation was seen. Depletion of accessory cells augmented the inhibition and soluble monoclonal anti-Fcγ receptor antibody completely abrogated the inhibition. Time-coarse experiments indicated that inhibition was real and not simply a change in the kinetics of the response. Complexes were only effective if added to cultures in the initial few hours after activation. Thus, cross-linking of B

lymphocyte Fcγ receptors by their specific ligand negatively regulates B lymphocyte differentiation but not proliferation at a particular stage in development.

The requirement for occupancy of B lymphocyte surface IgM in the negative regulation of B lymphocyte differentiation by Fcγ receptors was investigated. The degree of inhibition produced by complexes was directly proportional to the concentration of anti-mu used for triggering. Moreover, when B lymphocytes were activated with LPS, complexes only inhibited differentiation of B lymphocytes when antigen receptors were also occupied (TNP-specific B lymphocytes using either TNP-LPS or TNP on a separate carrier). Again, proliferation was not affected. Thus, the interaction between Fcγ receptors and surface IgM described above appears to be involved in the negative signalling of B lymphocytes by immune complexes bound to their Fcγ receptors.

Studies with monoclonal anti-Fcγ receptor antibodies (free of helper lymphokine) indicated that in soluble form they did not affect B lymphocyte responses. However, when on a Sepharose matrix they specifically inhibited B lymphocyte antibody production but had little effect on proliferation. Thus, provided that sufficient cross-linking can be obtained, the specific monoclonal antibody has effects similar to the specific ligand when bound to B lymphocyte Fcγ receptors.

At least 4 different monoclonal antibodies have been produced which react primarily with activated B lymphocytes. Spleen cells shown minimal numbers of reactive cells (0-15%), activated T lymphocytes are completely negative (0-1%) and activated B lymphocytes show reactivity (25-55%). Expression of the determinants is maximal 36-48 h after activation. The molecules recognized are candidates for receptors for growth and/or differentiation factors.

Significance to Biomedical Research and the Program of the Institute: Therapeutic manipulation of humoral immune responses both in cancer patients and other diseases ultimately depends on an understanding of the B lymphocyte membrane molecules which function as receptors for immunoregulatory signals (direct contact with other cells, Ig complexes, activating and deactivating lymphokines). Fcγ receptors have been identified as one such molecule, and it is expected that information concerning the function of other B lymphocyte membrane molecules will be forthcoming.

Proposed Course of Project: 1) Evaluate immune regulation of B lymphocyte function via Fcγ receptors in B lymphocyte subpopulations and various disease states including immunodeficient and autoimmune mice. 2) Evaluate covalently cross-linked monoclonal antibodies for effects on B lymphocyte function. Antibodies utilized will include those specific for Fcγ receptors, MHC class I and II molecules, 14D10, ThB, and surface IgD, alone and in combination. 3) Immunochemically and functionally characterize the molecules (recognized by the recently obtained hybridoma antibodies) which are expressed primarily on activated B lymphocytes. 4) Utilize these same monoclonal antibodies to define and characterize B lymphocyte subpopulations.

Publications:

Lamers, M. C., Heckford, S. E., and Dickler, H. B.: Hybridoma 2.4G2: Dissociation of the B lymphocyte triggering activity from the monoclonal anti-Fc receptor antibody. Molecular Immunol. 21:1237-1242, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05036-13 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*

Genetic Control of the Immune Response to Staphylococcal Nuclease

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principel Investigetor.) (Neme, title, leboratory, end institute affiliation)*

PI:	D. H. Sachs	Chief, Transplantation Biology Section	IB, NCI
Others:	R. J. Hodes	Chief, Immunotherapy Section	IB, NCI
	A. Finnegan	Guest Worker, Immunotherapy Section	IB, NCI
	C. A. Devaux	Visiting Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use stenderd unreduced type. Do not exceed the spece provided.)*

Antibodies directed against idiotypic determinants on anti-Staphylococcal nuclease antibodies from different mouse strains have been produced in rats and in pigs. The idiotypes are detected by ELISA assays and by the inhibition of antibody-mediated inactivation of nuclease. By screening a variety of strains and offspring from appropriate matings between strains for the presence of idiotypes and other markers, it has been shown that idiotype expression is linked to the heavy chain allotype markers. By means of an in vitro anti-TNP nuclease plaque-forming cell response, idiotypic markers have been demonstrated on T helper cells. Administration of anti-idiotypic antibodies to mice has been found to induce idiotype expression in the serum of these animals. This effect appears to involve T cells, since it is not observed in nude mice, and since idiotype-bearing T helper cells for in vitro anti-TNP nuclease response have been found in spleens from such treated animals. Several hybridomas reactive with nuclease and/or anti-idiotype have been produced. Syngeneic anti-idiotypes have also been produced and are presently being characterized in both antibody and T cell systems. Competitive binding studies are used to determine epitopes of nuclease as defined by available monoclonal antibodies. Examination of the kinetics of inhibition of nuclease with combinations of monoclonal antibodies is being used to determine mechanism of inhibition of catalytic activity of this enzyme. Site-directed mutagenesis of the nuclease gene has provided numerous point mutants of nuclease which are being studied for changes in immune reactivity.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: Staphylococcal nuclease (nuclease) is a protein enzyme, the immune response to which we have found to be under genetic control by H-2-linked Ir genes in the mouse. Because of the wealth of available data on structural and immunochemical properties of nuclease, it is an ideal model antigen for use in dissecting the mechanism of genetic control of immune responses. Such mechanistic studies at both the T cell and B cell levels are the objective of this project.

Methods Employed: 1) Antibodies to NASE have been prepared in groups of mice of a variety of strains (both high and low responders) differing in H-2 type or allotype or both. These antibodies have been purified by affinity chromatography. Inbred Lewis rats and miniature swine have been immunized with the purified anti-NASE antibodies obtained from immune ascites from A/J mice, SJL mice, B10.A(2R) mice, and BALB/c mice after immunization with NASE. Immunoabsorptions with normal immunoglobulins from the same strains have been used in order to determine whether or not antibodies reactive with the variable region (i.e., anti-idiotypic antibodies) have been produced.

2) Anti-idiotypic reactions have been quantified by hemagglutination, by ELISA, and by the inhibition of antibody-mediated enzyme inactivation. Such reactivities have been screened against anti-NASE antibody populations from the other strains of mice in order to determine the possible genetic linkage of idiotype to allotype and/or H-2 type.

3) Backcross animals have been screened for antibody levels and for allotype and idiotype expression in order to determine linkage of idiotype to other genetic markers.

4) Antibodies to nuclease have been separated on affinity columns into subpopulations directed against different determinants of nuclease. The reactions of anti-idiotypic antisera with these subpopulations have been characterized in order to determine new idiotypic markers.

5) Animals have been injected with purified anti-idiotype antibodies and the effect of this treatment on their subsequent expression of idiotype and anti-nuclease activity has been examined by means of spectrophotometric, ELISA and hemagglutination assays.

6) Anti-nuclease antisera and purified antinuclease antibodies have been examined by isoelectric focusing, using either protein stains or autoradiography employing ^{125}I-labeled nuclease or purified anti-idiotype.

7) An assay for secondary immune responses to TNP-nuclease in vitro has been developed. This assay has been used to examine the genetics of secondary responses to nuclease, as well as to assess the effects of anti-idiotype on the in vitro response and the cell level of expression of idiotype in this response.

8) Spleen cells from mice immunized with nuclease or treated with xenogeneic anti-idiotype have been fused to SP2/0 myeloma variant cells. Screening has been performed by an ELISA assay for binding either to nuclease or to anti-idiotype.

9) Assays for competitive binding of monoclonal antibodies to nuclease have been devised using the ELISA system. One monoclonal antibody is biotinylated and the effects of precoating nuclease plates with other monoclonal antibodies on subsequent binding of the biotinylated antibody are examined, with final development employing avidin-enzyme. In this way, epitopes of nuclease can be assessed, and correlated with the binding of the same monoclonal antibodies to nuclease fragments.

Major Findings: By two separate criteria, anti-idiotype antibodies have been obtained. 1) After exhaustive absorption with whole normal A/J ascites or repeated passages over affinity chromatography columns to which normal A/J ascites was bound, antibodies remained which reacted with A/J anti-NASE but not with B10.A anti-NASE antibody. Prior to absorption these rat antibodies contained precipitating antibodies to either type (A/J or C57BL/10) of antibody but no precipitating antibodies remained following absorption. 2) The rat anti-A/J anti-NASE antibodies prior to and following absorption reacted with the combining site of anti-NASE antibodies as evidenced by their ability to inhibit the anti-NASE antibody-mediated inactivation of NASE in an enzymatic assay.

Among (B10.AxA/J)xB10.A backcross animals the A/J anti-NASE idiotype was found to be linked ($p < .005$) to heavy chain allotype. However, a large recombination frequency was found (7-10 percent). The use of a very sensitive assay for allotype indicated that this recombination frequency was not the result of faulty allotypic typing. Also, progeny testing of the putative recombinant animals showed approximately 50 percent of the offspring to also have recombinant phenotypes, further indicating that these were true recombinant animals.

Injection of pig anti-idiotype antibodies into virgin mice has led to an increase in the level of idiotype in the mouse serum. That the reactive immunoglobulin molecules induced represented true idiotype and not anti-anti-idiotype was indicated by the fact that this induced idiotype was detectable using anti-idiotypic reagents produced in a variety of species. Similar treatment of nude mice did not lead to idiotype expression in the serum, suggesting that the effect may involve T cells. The idiotype detected in serum from the anti-idiotype treated mice was predominantly found on immunoglobulin molecules not detectably specific for nuclease. Treatment of such animals with nuclease led to an even greater increase in idiotype expression, and in this case there was also an increase in idiotype expression on anti-nuclease antibody molecules.

Spleen cells from anti-idiotype primed animals were found to provide T cell help in an in vitro anti-nuclease TNP plaque-forming cell response. This T cell help was equivalent to that obtained from nuclease-primed spleens. Anti-idiotype was found to inhibit this T cell help and in fact could eliminate the help if complement was added.

Isoelectrofocusing studies on anti-nuclease antibodies from a variety of strains have indicated that each strain has a characteristic spectrotype. Development of the isoelectrofocusing gels with ^{125}I-labeled anti-idiotype antibodies has shown a striking similarity in the clonotypes detected in all mice of a given strain, and there appears to be marked correlation with the expression of cross-reactive idiotypes as detected by serologic means.

Several hybridomas producing anti-nuclease antibodies have been prepared from immunized BALB/c, A/J and SJL mice immunized with nuclease. Also, several hybridomas producing idiotype without detectable antigen binding activity (ID') have been prepared from BALB/c mice treated with anti-idiotype.

Monoclonal antibodies to nuclease have been divided into at least three groups according to their epitope binding, as assessed by competitive inhibition of ELISA. Additional monoclonal antibodies have been produced and assessment of their epitope binding is in progress.

Significance to Biomedical Research and the Program of the Institute: Many of the antigens being studied in this laboratory are difficult to obtain in pure form (such as transplantation antigens and tumor antigens), and it is therefore important to have a well-characterized model protein on which initial experiments can be performed. In choosing models both for mechanistic studies and for studies in manipulation of the immune response, Staphylococcal nuclease is a particularly attractive protein. The immune response to this antigen has previously been shown in this laboratory to be under genetic control by an H-2-linked Ir gene.

The development of anti-idiotypic reagents directed toward antibodies under the control of an H-2-linked Ir gene provides another handle to study the mechanism of Ir gene function. These antibodies may help to determine whether the specificity of T cell recognition of antigens depends on similar variable region gene products to those responsible for B cell or antibody specificity. They may also lead to possible methods of controlling Ir gene expression. Finally, the detection of idiotypic determinants on T helper cells may provide a means of studying the elusive T cell receptor. Cloned lines of T helper cells bearing idiotype should provide a source for chemical characterization of the putative receptor.

Proposed Course of Project: The mechanism by which in vivo treatment with anti-idiotype leads to increased idiotype production will be pursued. Transfers of purified populations of T cells and B cells from immunized mice and anti-idiotype primed mice into irradiated recipients will be carried out. The effect of administration of inappropriate anti-idiotype antibodies to mice of various strains will be assessed. An examination of the control of idiotype expression at the T cell level will be attempted using the in vitro anti-TNP nuclease plaque-forming cell response.

Attempts to produce hybridomas secreting monoclonal anti-nuclease antibodies will be continued. These antibodies will be examined for expression of individual anti-nuclease idiotopes and will be used to produce monospecific anti-idiotope reagents for further characterization in in vitro and in vivo assays. In addition, attempts will be made to produce cloned T cell lines capable of providing T cell help for the in vitro anti-nuclease-TNP response.

Such lines will be examined both for anti-nuclease reactivity and reactivity with our anti-idiotypic reagents. Should such lines be established, attempts will be made to isolate and characterize the relevant T cell receptors.

Anti-idiotypic antibodies will be fractionated on immunoabsorbants bearing monoclonal anti-nuclease antibodies. These fractions, as well as syngeneic

anti-idiotypic reagents, will be examined for effects on humoral and cellular immunity to nuclease.

Numerous mutants of nuclease have recently been made available to us by Dr. David Shortle. These mutants are obtained by site-specific mutagenesis of the gene for Staphylococcal nuclease, and subsequent cloning and transfection of the mutated genes into E. Coli. Mutant nucleases will be isolated and characterized and used to help assess the binding specificity of both monoclonal antibodies and T cells specific for nuclease. The effects of selective mutants on Ir gene functions will also be assessed.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05038-13 I

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cell-Mediated Immunity to Modified Syngeneic Lymphocytes in Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. M. Shearer	Senior Investigator	IB, NCI
Others:	C. Via	Medical Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mouse spleen cells were modified with trinitrobenezene sulfonate (TNP), and the TNP-self modified cells were tested in two different ways. First, several monoclonal antibodies (mAbs) specific for class I H-2 antigens were tested for binding to TNP-modified spleen cells. Second, the modified cells were used as stimulator and target cells for in vitro tests for cytotoxic T lymphocyte (CTL) responses to TNP-self. A number of anti-H-2K^k antibodies but not mAbs of other specificities exhibited enhanced binding to cells that normally express K^k. Furthermore, these same mAbs bound to H-2^b cells modified with TNP. These results parallel the patterns of preferential CTL recognition in H-2^k mice and of crossreactive CTL in H-2^b mouse strains.

No work was performed on this project in FY 1985.

Project Description

Objectives: The primary objectives of this laboratory are to investigate the function of T lymphocytes, the role of self recognition, and the effects of major histocompatibility genes on the murine and human immune systems. These studies are being pursued using mouse and human leukocytes which are sensitized to autologous cells either modified with chemical agents (e.g. the trinitrophenyl group) or infected with viruses (e.g. influenza, cytomegalovirus). The murine TNP-self cytotoxic system serves as a basic model for testing many immunogenetic questions concerning the role of the MHC in immune regulation. The current specific objective of this project is to continue in the investigation of immunogenetic parameters associated with the in vitro generation of T-cell mediated immunity of murine cells to syngeneic cells modified with the TNP-group.

It is also our objective to use the hapten-self system as a sensitive indicator of T cell functions particularly in systems in which immune suppression is under study.

Methods Employed: For in vitro generation of CML activity mouse spleen cells were sensitized in vitro to autologous cells conjugated with trinitrobenzene sulfonic acid (TNP-self). The effector cells generated were assayed on the appropriate hapten conjugated or untreated ^{51}Cr-labelled target cells.

Major Findings: None.

Significance to Biomedical Research and the Program of the Institute: The project is of fundamental immunological interest in that it describes a major histocompatibility linked immunological phenomenon involving self recognition. The recognition of self MHC-coded structures in association with foreign antigenic determinants raises the possibility for self recognition as important for autoimmunity and for virally-infected autologous cells. The finding that products of the MHC are important for the antigenic complex recognized as well as for determining immune response potential provides a working hypothesis for bifunctional MHC control of disease susceptibility in those examples of HLA-associated diseases in man. The intricate immunoregulatory phenomena identified in the hapten-self cytotoxic system permits us to look for similar patterns in the virally infected murine and human models, and to attempt to determine whether such mechanisms could be operating in the immune systems of the intact individual, in either an infectious or neoplastic state.

Proposed Course of Project: Certain preparations of olive oils have been found in Spain that cause immunological and neurological problems in humans. It is considered that contaminants of this oil are inducing "modified self" immune reactions in these patients. We have obtained a sample of this oil and are attempting to induce "modified self" types of immune reactions in mice that have been exposed to these oils by skin painting, injection, and/or ingestion.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05050-11 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of Immunologically Relevant Cell Surface Phenomena

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. M. Segal	Senior Investigator	IB, NCI
Others:	P. Perez	Visiting Fellow	IB, NCI
	J. Bluestone	Laboratory Leader	IB, NCI
	R. Hoffman	Medical Staff Fellow	IB, NCI
	S. Shaw	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (a1) Minors ☐ (a2) Interviews

☒ (b) Human tissues — Human blood — B

☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. Cloned human cytotoxic cells (CTL) have been retargeted by using antibody heteroaggregates containing anti-T3 cross linked to anti-target cell antibodies. We have shown that by using this technique cloned CTL lose their normal specificity and can be made to lyse allogeneic and xenogeneic tumor cells, and even chicken red blood cells. These results suggest that effector cell retargetting could be used in vivo to treat neoplasms and other pathogens which express distinctive surface antigens.

2. In order to obtain effector cells which might be used in vivo to inflict cell mediated death upon tumor and other pathogenic cells, human peripheral blood leukocytes have been treated with anti-T3 containing antibody heteroaggregates and tested for the ability to lyse tumor cells expressing an antigen recognized by the second antibody of the heteroaggregate. Human peripheral blood T-cells when treated in this way are potent and specific mediators of target cell lysis. This is true without prior activation, but brief exposure to recombinant IL-2 rapidly increases the cytolytic activity to an even higher level. The effector cells in this case are $T8^+$.

Project Description

Objectives: (1) To investigate the molecular and cellular bases of the interactions of immunoglobulins with immune effector systems, (2) to study the relationship of antigenic recognition to these interactions, (3) to find methods by which these interactions can be artifically manipulated with the ultimate goal of increasing immune responses toward pathogens or neoplastic cells and decreasing autoimmune responses; and (4) to apply methods developed with the above studies to other immunologically related molecules and processes.

Methods Employed: Organic synthesis, gel filtration, ion exchange chromatography, polyacrylamide gel electrophoresis, complement fixation, radiolabeling of proteins, tissue culture, binding assays, cell and complement-mediated cytotoxicity assays, cell separations, flow microfluorometry, computer analyses.

Major Findings: We have previously shown that antibody heteroaggregates which contain anti-Fcγ receptor (FcγR) antibodies cross linked to nominan anti-target cell antibodies would bind to ADCC effector cells and cause them to specifically lyse target cells expressing determinants recognized by the anti-target cell antibody. Because cytotoxic T cells are more potent effectors against tumor targets than ADCC effector cells, we have used the same approach to retarget CTL.

Early studies in the mouse were unsuccessful because we had no antibodies against the T cell receptor. Cross linking of target cells to several cell surface components on mouse cloned CTL did not lead to target cell lysis. However, it has recently been shown in the human that the pan T cell marker, T3, is physically associated with the antigen-specific component of the T cell receptor, Ti. Moreover, Steve Shaw and collaborators have also demonstrated that hybridoma cells which make anti-T3 antibodies and which express these antibodies on their surfaces are lysed by cloned human CTL of a variety of specificity. Hybridomas producing other antibodies were not lysed. This suggested that anti-T3 linked the hybridoma directly to the T-cell receptor complex and triggered its own destruction. Implied in this observation is that anti-T3 containing antibody heteroaggregates would bind to human CTL and cause them to lyse any specified target cell.

We therefore switched to the human system. Anti-T3 was isolated and cross linked with anti $H\text{-}2K^k$ or anti-DNP antibodies. Four different anti-SB2 human CTL clones were treated with antibody heteroaggregates, a process which we term "franking", and tested for lysis against various target cells. First, the unfranked cells were shown to be actively lytic against M16, a $SB2^+$ lymphoblastoid cell line. Treatment with anti-T3 or with anti-T3 x $anti\text{-}K^k$ or anti-T3 x anti- DNP inhibited the lysis of M16 by the 4 CTL clones, thus confirming previous observations that anti-T3 inhibits CTL-mediated lysis and showing that the anti-T3 remained active within the heteroaggregates.

Next the CTL were tested for activity against a variety of targets. When cells were franked with anti-T3 x $anti\text{-}K^k$ they strongly lysed RDM4, an $H\text{-}2^k$ target, but not the $H\text{-}2^b$ target EL-4. They were also negative on chicken red blood cells (CRBC) and as mentioned, M16, the $SB2^+$ human line. When

franked with anti-T3 x anti-DNP the CTL failed to lyse M16, RDM4, EL4 or CRBC, but they did lyse all of these target cells when they were haptenated with TNP; free DNP-hapten in solution blocked lysis. These data show that by using antibody heteroaggregates to frank CTL clones, the specificity of the CTL can be altered. By using the appropriate antibody heteroaggregate we have been able to lyse every target cell to date which we have tested.

Finally we wanted to know whether direct linkage to the T-cell receptor complex was required for lysis or whether linkage of the target to any cell surface component on the effector would promote lysis. To test this, anti-T4 was linked to anti-K^k, and anti-DNP to anti-K^k. The CTL clones, which express T4, were then incubated with either M16 or RDM4 in the presence of anti-T4 x anti-K^k. We first showed that in the presence of anti-T4 x anti-K^k, the cloned CTL formed conjugates with both M16 and RDM4. However the cells lysed only their natural M16 target. Thus cross linking the target to the T4 subunit on the effector cell did not lead to target cell lysis. In a second experiment, the effector cell was lightly labeled with TNP. Again, cross linking of target cells to the TNP groups on the effector with anti-TNP x anti-K^k did not lead to lysis. Therefore we conclude that the target cell must be directly linked to the T cell receptor complex on the effector in order for lysis to occur.

Franked human PBL will mediate lysis of tumor targets. In order to use franked T cells to lyse pathogenic targets in vivo, we will need, optimally, autologous peripheral T-cells, and not T-cell clones as effectors. This is because cloned T cells are difficult to establish and obtain in large numbers. Human peripheral blood lymphocytes, however, are approxcimately 70-80% $T3^+$, and it was conceivable that many of these cells could mediate lysis when franked with anti-T3-containing heteroaggregates.

We therefore tested PBL from several donors against tumor targets in the presence of anti-T3 x anti-target cell heteroaggregates. In order to eliminate ADCC and NK activity, cells were first passed through a Sephadex G10 column and then treated with anti-leu 11b and complement. Such cells showed potent lytic activity against murine and human tumor targets, with little ADCC or NK activity. However, overnight incubation with crude lymphokine-containing supernatants caused an enhancement of this activity, usually in the 2-4 fold range. We then showed that pure recombinant IL-2 but not recombinant interferon γ- would mimic this effect. Kinetics studies showed that activation was rapid, occuring within 4-5 hr after addition of Il-2 to the culture medium. The cells mediating lysis were essentially all $T8^+$, $T4^-$ since treatment with anti-T8, but not anti-T4, + complement removed activity. In fact treatment with anti-T4 + complement substantially increased the activity of the franked T-effector cells, as expected since T cells are usually about 2/3 $T4^+$, 1/3 $T8^+$, with very few containing neither or both antigens. The lytic activity of the $T4^-$ cells was similar to that obtained with cloned T cells, that is significant lysis of tumor targets occurred at a 1:1 effector:target ratio.

These studies suggest that franked autologous PBL could be used as potent anti-tumor effector cells in vivo, especially after a few hours incubation with IL-2.

Significance to Biomedical Research and the Program of the Institute: The recognition of foreign substances by antibodies elicits a number of reactions which normally leads to their elimination from the body. The purpose of this project is to examine the molecular events which occur as a result of antigenic recognition. It is hoped that these studies will enhance our understanding of these processes and allow us to better control immune reactions. An ultimate goal is to enhance the immune response toward neoplastic cells.

Proposed Course of Project: We plan to extend these studies in vitro using specific anti-human tumor monoclonal antibodies to see if we can obtain tumor-specific lysis by franked human PBL. We will collaborate with several laboratories on this project. Dr. Frank Cuttitta (DCT, COP, NNMC) has already given us antibodies against small and large cell lung carcinomas plus appropriate cell lines to test these on. We have also received an anti-Reed-Sternberg monoclonal from Dr. Eric Groves (DCT MB) and weakly positive cells, as a model of Hodgkins disease. Finally we are currently initiating collaborations with other laboratories at NIH to develop in vitro cytotoxic systems using franked PBL.

To facilitate these studies we are currently using anti-T3 coupled to avidin to frank T cells. Any specificity can then easily be conferred upon those cells by incubating them with biotinylated anti-tumor antibodies.

Our next steps will be to test these anti-tumor systems in vivo, first in nude mice, and then perhaps as phase I trials in cancer patients. Dr. John Wunderlich has already agreed to collaborate with the mouse studies, and I am currently speaking with investigators at the NIH who have already established clinical protocols to see if and when experiments in patients could be initiated.

Publications:

Levy, R. B., Dower, S. K., Shearer, G. M. and Segal, D. M.: Trinitrophenyl modification of H-2^k and H-2^b spleen cells results in enhanced serological detection of K^k-like determinants. J. Exp. Med., 159:1464-1472 (1984).

Katona, I. M., Urban, J. F., Titus, J. M., Stephany, D. A., Segal, D. M. and Finkelman, F. D.: Characterization of murine lymphocyte IgE receptors by flow microfluorometry. J. Immunol., 133:1521-1528 (1984).

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Dower, S. K., and Segal, D. M.: Interaction of monoclonal antibodies with MHC class I antigens on mouse spleen cells. II. Levels of expression of H-2K, H-2D, and H-2L in different mouse strains. J. Immunol., 134:431-435 (1985).

Perez, P., Bluestone, J. A., Stephany, D. A., and Segal, D. M.: Quantitative measurements of the specificity and kinetics of conjugate formation between cloned cytotoxic T lymphocytes and splenic target cells by dual parameter flow cytometry. J. Immunol., 134:478-485 (1985).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05058-10 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunoregulation by Anti-idiotype Antibodies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. B. Dickler	Senior Investigator	IB, NCI
Others:	G. Berrebi	Medical Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

Dr. Seth Pincus, University of Utah

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to characterize the mechanisms by which anti-idiotype antibodies regulate immune responses and lymphocyte function. A system has been developed in which, for the first time, soluble antibody responses to the synthetic polypeptide (T,G)-A--L can be generated and detected in vitro using antigen-primed lymph node cells. Responses are antigen dependent and specific, and H-2 linked Ir gene regulated. Antibodies specific for the idiotypes of anti-(T,G)-A--L antibodies induce antigen-independent anti-(T,G)-A--L antibody responses. These responses are specific at the levels of the anti-idiotype reagent, the antigen-priming, and the antibody produced. The anti-idiotype antibodies stimulate function from antigen-primed T lymphocytes in the form of soluble helper lymphokines, and function from both primed and unprimed B cells in the form of specific antibody secretion. Unprimed B cells, in addition to anti-idiotype, require either primed T cells or idiotype or unrelated antibody complexes to be present in order to obtain function. Responses to anti-idiotype antibodies, in contrast to those to antigen, appear not to be regulated by Ir genes. A monoclonal anti-idiotype which reacts with a public idiotope present on the majority of anti-(T,G)-A--L antibodies from mice of the IgCH b haplotype e.g., B10 but absent on such antibodies from mice of the IgCH a haplotype e.g., Balb.B has been obtained. Expression of this idiotype has been assessed in anti-(T,G)-A--L responses utilizing T and B lymphocyte mixtures prepared with cells from idiotype positive and negative strains in all combinations. No evidence was obtained for an idiotype-regulating T helper lymphocyte. The monoclonal anti-idiotype in soluble form failed to stimulate anti-(T,G)-A--L responses in vitro.

Project Description

Objectives: Characterization of the mechanisms by which anti-idiotype antibodies regulate immune responses and lymphocyte function.

Methods Employed: An in vitro microculture system using antigen-primed lymph node cells is utilized. Antibody responses are measured using an enzyme-linked immunoabsorbant assay (Elisa) with antigen-coated plastic plates. Immunoregulation is studied by manipulation of the conditions of interaction, and by immunologic and chemical modifications of both the lymphocytes and reagents. Anti-idiotype antibody is prepared by immunization of Lewis rats with antigen-affinity purified murine anti-(T,G)-A--L antibodies with subsequent adsorption on normal mouse immunoglobulins. Anti-idiotype activity can be measured by inhibition of binding of anti-(T,G)-A--L antibodies to the antigen, or by direct binding to the idiotype. Genetic studies are carried out utilizing inbred, congenic, and/or recombinant mouse strains. Lymphocyte subpopulations are prepared by a variety of techniques including antibody and complement mediated lysis, antibody affinity plates, density gradients, and adherence columns. Other techniques employed include flow microfluorometry and production and purification of monoclonal antibodies. A monoclonal anti-(T,G)-A--L anti-idiotype antibody was prepared by standard hybridoma technology using immune Lewis rat spleen cells from a Lewis rat immunized as described above.

Major Findings: Using a microculture system we have been able to obtain antigen-specific, T lymphocyte dependent responses to (T,G)-A--L in vitro. This is the first known success at obtaining a soluble antibody response in vitro to an Ir gene regulated antigen. The characteristics of the system are as follows: 1) In order to detect such responses it is necessary to wash the antigen out of the cultures after 3 days and use a very sensitive detection system (Elisa); 2) Primed T lymphocytes are required; 3) The response is antigen-dependent and specific; 4) The response is under Ir gene control and the Ir genes are phenotypically expressed by B lymphocytes and/or accessory cells; 5) T and B lymphocytes can cooperate to produce these responses if they are H-2 identical even if non-H-2 genes are different; 6) Responses are highly reproducible and levels of antibody are 100-500 ng/ml.

We have evaluated the effects of anti-idiotype reagents in this system. It was found that antigen-primed lymph node cells responded to anti-idiotype by the production of antibody in the absence of antigen. This antigen-independant response was specific at the level of the anti-idiotype reagent in that other reagents including normal rat serum, rat anti-mouse IgG, rat anti-mouse IgM and rat anti-nuclease anti-idiotype were not active. Moreover, idiotype affinity-purified anti-idiotype was active. The response was also specific at the level of priming in that cells primed to CFA or ovalbumin would not respond to anti-(T,G)-A--L anti-idiotype. Finally, it was specific at the level of the antibody produced since antibodies against other antigens were not induced.

The cellular site of action of the antigen-independent anti-idiotype induced anti-(T,G)-A--L response was evaluated. Rigorously B lymphocyte depleted antigen-primed T lymphocytes together with equal numbers of unprimed B lymphocytes (unprimed lymphocytes, B or T, would not respond to anti-idiotype)

responded to anti-idiotype, suggesting that the anti-idiotype was provoking function from T helper lymphocytes. This was directly shown by stimulating antigen-primed T cells for 3 days with anti-idiotype and collecting the supernatant. This supernatant (but not controls) contained soluble helper lymphokines which allowed unprimed B cells to produce specific antibody when stimulated with anti-idiotype (but not controls). Thus, the anti-idiotype directly triggers both T and B lymphocytes. The T lymphocytes which respond to anti-idiotype have been characterized as Lyt 1 positive, Lyt 2 negative. Responses to the anti-idiotype do not appear to be regulated by Ir genes. Thus, mixtures of (Responder x Nonresponder)F_1 antigen-primed T lymphocytes with Nonresponder B plus accessory cells respond to anti-idiotype but not to the antigen.

Antigen-primed B lymphocytes rigorously depleted of T cells specifically respond to anti-idiotype by secretion of anti-(T,G)-A--L antibodies. This response was shown to be specific by a variety of criteria. This stimulation of function by anti-idiotype appears to involve idiotype-anti-idiotype complexes because normal B cells respond to neither idiotype or anti-idiotype but do respond to a mixture of the two. Moreover, Fcγ receptors appeared to be involved in that normal B lymphocytes would also respond in the presence of anti-idiotype plus unrelated antibody complexes.

A rat γ2b,k monoclonal antibody produced by a hybridoma (I-9) has been obtained in collaboration with Dr. Seth Pincus, University of Utah. This antibody reacts with a public idiotope present on the majority of anti-(T,G)-A--L antibodies from mice of the IgCH b haplotype but not on such antibodies from mice of the IgCH a haplotype. The idiotype appears to be near the combining site. In soluble form this monoclonal anti-idiotype has failed to stimulate anti-(T,G)-A--L responses in vitro.

Anti-(T,G)-A--L responses were obtained in vitro from mixtures of T and B lymphocytes prepared in all combinations of cells from idiotype positive and negative strains. Idiotype expression was not affected by the IgCH haplotype of the T helper lymphocytes. Thus, no evidence was obtained for the existance of an idiotype regulating T helper lymphoycte.

In order to evaluate supernatants from T lymphocytes stimulated by anti-idiotype for helper function a new assay has been developed. Unprimed B lymphocytes are incubated with primed helper T lymphocytes and antigen for 48 hrs. The T lymphocytes and antigen are then eliminated and antigen-specific responses are obtained in the presence but not the absence of helper lymphokines. This assay will allow evaluation of the nature of helper lymphokines generated by the interaction of anti-idiotype and helper T lymphocytes.

Significance to Biomedical Research and the Program of the Institute: An understanding of the regulation of the immune response could lead to new forms of therapy for human diseases including cancer. Specifically, the findings outlined here indicate it is possible to trigger antigen-specific immune function of lymphocytes in the absence of antigen using anti-idiotype reagents. This raises the possibility of the use of such reagents themselves as therapeutic modalities.

Proposed Course of Project: A) Determine the requirements for triggering antigen-independent responses using monoclonal anti-idiotype including: 1) using the antibody in a form which will produce cross-linking of receptors (Sepharose matrix or covalent cross-linking); 2) providing various forms of T lymphocyte help including helper T lymphocytes which recognize the monoclonal antibody and various lymphokine preparations; and 3) avoiding Fcγ receptor inhibitory effects. B) Determine the mechanisms by which injection of the idiotype in vivo causes expression of the idiotype in an idiotype negative animal.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05062-10 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Application of Rapid Flow Microfluorometry to Cell Biology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. R. Wunderlich	Senior Investigator	IB, NCI
	S. O. Sharrow	Chemist	IB, NCI
Others:	Members of the Immunology Branch (see text)		

COOPERATING UNITS *(if any)* M. T. Lotze, Surgery Branch, NCI; S. Rosenberg, Chief, Surgery Br., NCI; D. Longo, Medicine Branch, NCI; A. Kruisbeek, Medicine Branch, NCI; R. Fico, DCRT; A. Schultz, DCRT; W. I. Frels, University of Utah; and L. Hood, Cal. Tech.

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	0.1	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

x

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Using rapid flow microfluorometry (FMF) for analysis and sorting of cells, aspects of the following projects have been supported during the previous year: (1) stoichiometry and kinetics of cytotoxic cell binding to target cells, (2) effects of human recombinant IL 2 therapy on peripheral blood lymphocytes, (3) characterization of the specificities of monoclonal antibodies against major histocompatibility complex (MHC) determinants, (4) function of transfected MHC genes, and (5) monitoring the effectiveness of in vivo immune cell depletion or reconstitution associated with various types of therapy. In addition, the capabilities of the FMF facility have been expanded by 1) increasing the number of parameters which can be analyzed on each cell, (2) adding a flow cytometer analyzer, and (3) increasing the availability and capability of data processing.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives: The objective of this project is to utilize rapid flow microfluorometry (FMF) to study selected aspects of important problems which would be impossible or extremely difficult to pursue without such technical support.

Methods Employed: Cells have been both analyzed and separated by rapid flow microfluorometry (Fluorescent-Activated Cell Sorter (FACS) and FACS analyzer Becton-Dickinson Electronics Laboratory, Mountain View, CA). These studies involve preliminary treatment of suspensions of viable, dispersed cells with purified, well-characterized fluorescent reagents.

Major Findings: This report summarizes the major thrust of each overall project emphasizing those aspects most heavily supported by the use of rapid flow microfluorometry (FMF).

Segal, Perez, Stephany, Bluestone and colleagues. Previously, this group established a quantitative method for measuring cell:cell binding (conjugate formation) by dual laser FMF. The model consisted of antibody-treated spleen cells bound to macrophages through macrophage Fc receptors for immunoglobulin. The two interacting cell types were labeled with different fluorchromes prior to mixing. Models for cell conjugate formation suitable for FMF analysis have now been extended to cell binding mediated by antibody heteroaggregates and to binding of cloned cytotoxic T lymphocytes (CTL) to sensitive target cells. Heteroaggregates have been generated by covalantly cross-linking antibodies against immunoglobulin Fc receptors (FcR) to antibodies against target cell antigens. These antibody complexes mediate antigen-specific binding and lysis of target cells by a variety of different types of effector cells, including FcR^+ lymphocytes, macrophage cell lines, and human neutrophils. The binding of effector cells to target cells by heteroaggregates is less susceptible to nonspecific inhibition by circulating immunoglobulin, because the FcR binding is mediated by a high affinity antibody combining site rather than by the low affinity interaction of immunoglobulin Fc fragments and FcR. Parallel studies of CTL-target cell interactions have revealed that cell:cell binding follows first order kinetics, that effector cells are recycled, and that nearly all conjugates contain one effector and one target cell, irrespective of the starting cell ratio.

Lotze, Rosenberg, and colleagues. This group, which previously studied the effects of treating patients with interleukin-2 (IL-2) derived from the Jurkat cell line, has recently used recombinant IL-2 in phase one clinical trials. FMF has again been used to monitor the effects of treatment on peripheral blood lymphocyte subsets. With bolus infusion, the relative and absolute levels of all lymphocyte subclasses decreased: T lymphocytes ($Leu3^+$ and $Leu2^+$ cells), N/K Cells ($Leu7^+$ cells) and B cells/monocytes ($Leu10^+$ cells). A rebound occurred at 24 hours, resulting in increased numbers of all lymphocyte subclasses by 48 hours. Continuous IL-2 infusion resulted in early decreases in lymphocytes and increases in macrophages. After cessation of therapy, there was a rebound increase in lymphocytes of both the Leu2 and Leu3 subclasses. The rebound effect included an unusually large level of cells expressing IL-2 receptors - up to 25% of the mononuclear cells.

Pescovitz, Sachs, and Shaw. These groups have used flow cytometry for characterizing the specificities of monoclonal antibodies (mAbs) against MHC determinants and lymphocyte subsets in swine and man. Thus, Pescovitz and Sachs have identified anti swine cell mAbs which recognize helper (PT4) and cytotoxic (PT8) T cell subsets, peripheral B cells and 60% of thymocytes (76-7-4), and macrophages and granulocytes (74-22-15 and 76-5-28). Shaw has studied the specificity of a large panel of monoclonal anti-Ia antibodies using HLA deletion mutant target cells, and he has identified patterns of reactivity consistant with three Ia-subregions in man (DR, DC, and SB). Distinct advantages of FMF, compared to alternative procedures such as ELISA assays, have been apparent in these studies. For example, cell-fixation artifacts and non specific binding of reagents to wells, which are common problems with ELISA assays, have been avoided by use of FMF. Moreover, an unusual feature of FMF has been helpful in identifying mAb specificities. From past experience, staining profiles (fluorescence distribution histograms) have been identified which are characteristic of particular T-lymphocyte subclasses, such as those generally associated with helper functions ($L3T4^+$ mouse cells, OKT4 human cells, and PT4 pig cells). Preliminary identification of mAb specificity often can be made from the staining profile alone, and this capability helped identify the specificities of PT4 and PT8 mAbs.

D. Singer, Frels, Bluestone, Hodes, and colleagues. This group has utilized FMF to study the expression of a porcine (pig) class I MHC gene introduced into the genome of a C57BL/10 mouse by microinjection of the xenogeneic gene into fertilized eggs. Progeny of the transgeneic mice bred to normal C57BL/10 mates express porcine MHC antigens on cells from a variety of tissues and express the porcine MHC molecules as functional transplantation antigens (C57BL/10 transgeneic mouse skin is rejected by normal C57BL/10 mice). The xenogeneic gene is transmitted in accordance with Mendelian inheritance and appears to be integrated as one or a few copies into a single site of the host genome. The sensitivity of FMF has been an important aspect of the work, because porcine antigens are expressed at low levels on transgeneic mouse cells. Thus, FMF has provided a way to identify mice bearing the heterozygous xenogeneic gene and has been a useful means to study the biology related to its expression.

Sharrow, Sachs, Hood, and colleagues. Previously, Sharrow and Sachs identified 2 monoclonal antibodies against mouse class I H-2 determinants, which cross reacted with determinants controlled by genes to the right of $H\text{-}2^d$ on chromosome 17. This group has recently identified at least one gene, which may be associated with the cross-reactive determinant. The gene is Q6, which is in the Qa-2 region of the MHC. The gene product has been selectively analyzed with FMF by using mouse fibroblasts bearing a hybrid $Q8/L^d$ gene, introduced by gene transfer.

Ildstad, Sachs, Kruisbeek, Longo, and colleagues. FMF has been utilized by this group for phenotyping cells, in order to detect low levels of specific types of cells in bone marrow chimeras or in animals immunosuppressed by therapy with monoclonal antibodies. Thus, Ildstad and Sachs have accurately determined low percentages of rat cells in various organs of lethally irradiated mice reconstituted with mouse and rat cells. Specific tolerance of rat skin grafts and hyporeactivity in vitro to rat cells by the reconstituted mice has

been associated with low levels, if any, of donor-type cells in lymphoid tissues. In a separate study of antigen presenting cell function, which is an important component of normal immune competence, Kruisbeek and Longo have monitered the depletion and reappearance of Ia-positive cells, following treatment of mice with monoclonal antibodies against these determinants. Reappearance of antigen presenting cell function parallels the reappearance of Ia-positive cells.

Fico, Sharrow, Stephany, Schultz and colleagues. Major changes have been made this year in the FMF facility. A dye laser has been added to the FACS II instrument, in order to increase the number of wave lengths available for exciting fluorochromes. The number of parameters which can be analyzed on each cell has been increased to four, which can be chosen from three fluorescence signals and two light scatter signals (forward and 90° angle scatter). A flow cytometer analyzer has been added in order to increase investigators access to FMF. Individual investigators, about 12 to date, have been trained to use the instrument, which is less versatile and sensitive than the FACS II but is more user-friendly. To increase the efficiency of the laboratory, data processing has been converted to a new operating system (RSX II M), which will allow simultaneous data acquisition and data analysis.

Significance to Biomedical Research and the Program of the Institute:
Analysis of cell:cell conjugates by FMF has been extended to two types of effector cell-target cell interactions particularly relevant to anti tumor mechanisms. Cytotoxic T lymphocytes constitute one of the major arms of cytotoxic cell anti tumor responses, and artificial bridging of cytotoxic lymphocytes to tumor cells by heteroaggregated antibodies is one of the newest, most promising approaches to immunotherapy. Because target cell lysis by effector cells involves mechanisms additional to those required for target cell binding, it is important to be able to distinguish experimentally between the two steps. Cell:cell conjugate analysis offers this feature.

Flow cytometry has been particularly useful in monitoring treatment-induced changes in patients' peripheral blood cells, because FMF identifies subpopulations of cells whose expression of cell surface differentiation antigens (phenotype) has been associated with certain biologic functions. The advantage of FMF is the relative simplicity and quantitative nature of the test, as illustrated by ongoing studies of NCI Surgery Branch patients treated with interleukin-2.

During the last year FMF has been used frequently for studies involving DNA transfected cells and for testing the antigenic specificity of monoclonal antibodies. FMF has been a distinct asset to molecular biology studies involving cells transfected with MHC genes, because low levels of gene product are accurately detected at the single cell level and because a wide variety of well-characterized monoclonal antibodies against MHC determinants are available. Results of these studies will advance our understanding of the role of MHC products in immune responses against MHC-restricted antigens (e.g., tumor antigens) and in the development of immunologic tolerance.

FMF has also provided a strong source of support for studies of the major histocompatibility gene complex, whose products are well known to have a major

role in immunologic recognition of foreign cells such as antigenic tumors and bone marrow grafts. These projects have included the study of MHC determinants on cells from transfected tissue culture lines or from transgeneic mice. The advantages of FMF have included (1) multiparameter analysis which simultaneously detects different determinants at the single cell level, (2) relatively precise quantitation, and (3) a relative lack of artifacts compared, for example, to ELISA assays.

Proposed Course of Project: As in the past, FMF will be used for selected, appropriate projects.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05064-09 I

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic Control of the Immune Response In Vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Singer	Senior Investigator	IB, NCI
Others:	J. Bluestone	Laboratory Leader	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The possibility was investigated that class I MHC antigen can serve both as restriction elements and as nominal antigens. It was found that T cell hybrids can be generated which are self-Ia restricted but which are specific for class I MHC alloantigens, suggesting that they recognize a composite determinant composed of class I and class II MHC molecules. T cell recognition of such composite determinants is inhibitable by anti-L3T4 and anti-Ia antibodies. Thus, these results suggest that class I MHC antigens can be seen as nominal antigens in association with self-Ia restriction determinants.

GPO 914-918

Project Description

Objectives: The major objective of this project is to examine composite determinants composed of class I and class II MHC molecules.

Methods Employed: T cell hybrids were constructed from cell populations that were stimulated with class I alloantigens. The specificity of the T cell hybrids so constructed is determined by their secretion of Interleukin-2 in response to ligand. Secreted Interleukin-2 is assayed by its ability to maintain the growth of an IL-2 dependent cell line, HT-2.

Major Findings: T cell hybrids were identified with 3 distinct specificities for: (a) allogeneic class I MHC determinants with no apparent class II restriction; (b) self-class II MHC determinants; and most interestingly (c) allogeneic class I + self-class II MHC determinants. Regardless of their specificity, all the T-hybrids isolated so far are $L3T4^{+}$ $Lyt2^{-}$. These findings support the existence of composite MHC determinants.

Significance to Biomedical Research and the Program of the Institute: The identification of composite determinants makes it possible to explore their role in inducing rejection of tissue grafts and tumors.

Proposed Course of the Project: Our immediate goals are to assess the role of composite determinants in self-tolerance and allograft rejection.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05067-10 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanisms of Human In Vitro Cellular Immune Responses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Shaw	Senior Investigator	IB, NCI
Others:	W. E. Biddison	Senior Investigator	NI, NINCDS
	R. Hoffman	Medical Staff Fellow	IB, NCI
	M. Sanchez-Perez	Visiting Fellow	IB, NCI
	J. Bluestone	Laboratory Leader	IB, NCI
	O. Leo	Visiting Fellow	IB, NCI
	S. Sharrow	Chemist	IB, NCI

COOPERATING UNITS *(if any)*

P. Gallop and T. Springer, Harvard Medical School, Boston, MA

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.6	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies are continuing on the process of recognition of foreign antigen by human T cells, particularly with respect to the role of T cell surface molecules in cytotoxic T cell (CTL) interaction. Recognition of the DP antigens has been used as a model system using a panel of DPw2-specific CTL clones. Visible conjugate formation between effector and target has been analyzed by flow cytometry using a new dye combination we have identified which avoids covalent modification of the cell surface. In our system there is strong antigen-nonspecific conjugate formation and the specificity of killing is determined at the level of triggering. We believe the antigen-nonspecific conjugates to be a useful model system in which to understand the role of monomorphic cell surface molecules (e.g., LFA-1, LFA-2, LFA-3) in interactions of lymphoid cells. Studies of the T3 molecule have confirmed its unique role in triggering T cells. Anti-T3 immobilized on assay plates show a unique enhancement of their ability to inhibit CML; and anti-T3 expressing hybridomas trigger their own lysis by DPw2-specific CTL. Surprisingly, anti-LFA-1 inhibits anti-T3 triggered lysis, suggesting that LFA-1 plays a crucial role even in this xenogeneic interaction which bypasses antigen-specific steps.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: The primary objectives of this laboratory are to investigate the function of T lymphocytes, the role of HLA antigens in T cell recognition, and the mechanism by which these HLA genes control immune responses. The hypotheses generated draw heavily from the precedents in animal models, particularly those in the mouse, but the experimental work is restricted to human studies. Because of the ethical and logistical considerations which limit in vivo studies in humans, it has been (and is) crucial to develop good in vitro models of human immune responses. The model system used most extensively has been cytotoxic and proliferative T cell responses to the HLA-DP antigens on allogeneic cells.

Methods Employed: Human PBL are obtained from donors by phlebotomy or batch leukapheresis; mononuclear cells are prepared by density separation, and cryopreserved. Analysis of the HLA markers on the donors cells is performed by microcytotoxicity testing under contract NO1CB33935. Responses to the DP antigens have been generated by priming T cells between allogeneic donors matched for all known HLA antigens except DP. For higher resolution analysis of the specificity and function of individual T cells, single cell cloning has peen performed by limiting dilution and subsequent expansion in the presence of specific antigen and TCGF. These clones are cryopreserved and can be used repeatedly over long periods of time. Cells are assayed for cytotoxicity in short term ^{51}Cr release assays using as targets T lymphoblasts or lymphoblastoid B cell lines. Conjugate formation is analyzed either by assessing functional conjugates in an assay involving dispersion of cells into a viscous media (which prevents formation of new conjugates) or by enumerating visible conjugates by flow microfluorometry using a new dye combination identified as described below.

Major Findings: T cell responses to the HLA-DP antigens provide an optimal experimental system in which to dissect out functional components of the cellular interactions which occur between antigen-specific T cells and antigen-positive cells. It is plausible that many of the T cell differentiation antigens now being defined on T cells by monoclonal antibodies are functionally involved in antigen recognition by T cells. In collaboration with Dr. W. E. Biddison, we have analyzed the importance of these molecules in the interaction between DP-specific cytotoxic cells and their targets by studies of inhibition with monoclonal antibodies against these molecules. Ten DP2-specific CTL clones previously cloned by limiting dilution have been further characterized.

We have extended our previous analysis of conjugate formation of these clones by studying visible conjugates as well as functional conjugates (discussed in last years annual report). We have modified an approach recently described by Dr. David Segal for the enumeration of conjugates by flow microfluorometry. This modification uses intracellular fluorescent dyes rather than dyes covalently bound to the cell surface; this modification may be an important alternative since covalent modification of cell surfaces can cause aberrant recognition by T cells. Furthermore, this modification was made in order to be able to use the Becton Dickinson Analyzer recently purchased by the branch, which excites at a single wavelength. After evaluation of an extensive panel of potential dyes, sulfo-fluorescein diacetate and hydroethidine were found to be a suitable combination of dyes.

Using this approach it has become apparent that most of the antigen specificity in this model system is based on the specificity of triggering, not of conjugate formation. Strong conjugates are formed between DPw2-specific CTL and 'target' lymphoblastoid cell lines whether or not they express the DPw2 molecule. Such conjugate formation occurs rapidly (peaks at 6 minutes) at physiological temperature, but also proceeds at 20°C and more slowly at 4°C. Both antigen-specific and antigen-nonspecific conjugates appear to use the LFA-1, LFA-2 (T11) and LFA-3 molecules, since their formation is substantially inhibited in the presence of monoclonal antibodies specific for those molecules. But conjugate formation is not markedly inhibited by monoclonal antibodies specific for T3, DP or T4. We postulate that transient antigen-independent conjugate formation allows intimate approximation of membranes of the effector and potential target. During this encounter, the antigen-specific receptor (Ti) on the effector can 'sample' the surface antigens on the target. Low concentrations of surface antigen have a good likelihood of encountering Ti during such an encounter.

The finding that anti-T4 does not inhibit visible conjugate formation was in contrast to our previous finding that it does inhibit functional conjugate formation. This contrast was confirmed in direct comparisons of the two assay systems and suggests that anti-T4 acts in some fashion distinct from gross physical approximation of the cells. Two possible roles are considered particularly attractive. First, intimate approximation of membranes (at a distance optimal for antigen-Ti interaction) would enhance the efficiency of triggering. Alternately (or additionally), interaction of T4 with a non-polymorphic part of Ia would enhance the concentration of Ia in the zone of contact since Ia is mobile in the membrane. This increased concentration of Ia should enhance Ia interaction with Ti (if one assumes that Ti and T4 can bind Ia simultaneously).

We have explored an alternate approach to antibody inhibition -- exposing the cells to antibody which coats the bottom of the plastic well in which the cytotoxic reaction occurs. Exposure to such immobilized antibody differs from exposure to antibody in solution in critical ways including: 1) the antibody is presented in a form which is highly multivalent; and 2) the effect of the antibody is removed from the site of interaction between the effector and target. The most consistent difference observed between this approach and the conventional one is the enhanced capacity of anti-T3 antibody to inhibit CML. Although antibodies against most structures (anti-LFA-2, anti-T4, anti-Ia) inhibit no better when immobilized, anti-T3 is generally 10-30 fold more efficient in inhibiting when immobilized. Pending the outcome of additional studies, we propose two plausible explanations for the enhanced inhibition by anti-T3: 1) the T3 molecule may redistribute more rapidly or 2) the immobilized anti-T3 may be more potent because of the extensive multivalency, which results in T3 aggregation on the effector and triggering.

The hypothesis of anti-T3 induced triggering was tested directly, by analyzing the ability of the DPw2-specific CTL clones to lyse xenogeneic cells expressing the anti-T3 antibody (e.g., the OKT3 hybridoma). The OKT3 hybridoma was very efficiently lysed, but hybridomas expressing on their surface antibody against other molecules were not lysed. These data suggest that cell-bound anti-T3 can substitute for antigen in triggering cytotoxic activity.

The mechanism of anti-T3 triggered lysis was further explored by studies of monoclonal antibody inhibition of this mode of lysis. It was inhibited by anti-T3 antibody (at high concentration) and by anti-LFA-1 antibody, but not by anti-LFA-2, anti-LFA-3 or anti-T4. This indicates that LFA-1 continues to play an important role even in this pathway which bypasses requirements for antigen and Ti.

Significance to Biomedical Research and the Program of the Institute: The problems addressed in this project are central to the understanding of how the immune system surveys the body to detect foreign antigen and to eliminate cells which express those foreign antigens. This surveillance system is thought to be important not only in dealing with microbial pathogens but also in detection and destruction of neoplastic changes. In particular, the complex modes of cytotoxicity seen with CTL clones are similar to modes of natural killer activity, which has been postulated to be important in tumor immunity. Furthermore, these studies promise to clarify the relevance of genetic differences between individuals in their susceptibility to infectious, neoplastic and autoimmune diseases.

Proposed Course of the Project: Future studies will extend the present results in several areas. With respect to conjugate formation, we will continue to explore the details of the kinetics of conjugate formation and the agents which modify that conjugate formation or the lytic efficiency of the conjugates formed. In particular, we will seek to test two working hypotheses: 1) that critical molecules are focused into the region of interaction (i.e., their concentration is increased in the area of contact between the two cells); and 2) that the loss of the bond between effector and target involves an active process of dissociation even in the case of nonspecific or non-lytic conjugates. We will also seek alternate ways of detecting triggering (perhaps by measuring some event in cytotoxic granule release) so that this process can be studied without a target cell as a readout.

Publications:

Biddison, W. E. and Shaw, S.: Antibodies to the T4 (Leu 3) molecule block CTL-target cell binding but do not inhibit the lytic phase of cytotoxicity. In: Cantor, H., and Chess, L.: Regulation of the immune system Vol 18. Alan R. Liss, NY. p. 221-230, 1984.

Biddison, W. E., and Shaw, S.: SB-specific CTL clones exhibit functional heterogeneity in their susceptibility to blocking by anti-T3 and anti-T4 antibodies. In: Feldman, M. (Ed.) Human T cell clones: a new approach to immune regulation. Humana Press, London, in press, 1984.

Hoffman, R. W., Bluestone, J. A., Leo, O., and Shaw, S.: Lysis of anti-T3 bearing murine hybridoma cells by human allospecific cytotoxic T cell clones and inhibition of that lysis by anti-T3 and anti-LFA-1. J. Immunol., in press, 1985.

Hoffman, R. W., Quinones, R. R., and Shaw, S.: Anti-T3 antibody both activates and inhibits the cytotoxic activity of human T cell clones. In: Meuer (Ed.) Proceedings of International Workshop on Human T cell clones. Behringwerke, in press, 1985.

Perez, P., Hoffman, R. W., Shaw, S., Bluestone, J. A., and Segal D. M.: Specific targeting of CTL by anti-T3 linked to anti-target cell antibody. Nature (Lond.), in press.

Shaw, S.: Book review. Methods in enzymology. Volume 108. Immunochemical Techniques Part G. Anal. Biochem. In press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05069-09 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Expression of Ia Antigens on Functional Cell Subpopulations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Hodes	Chief, Immunotherapy Section	IB, NCI
Others:	B. Needleman	Medical Staff Fellow	IB, NCI
	D. H. Sachs	Chief, Transp. Biol. Sec.	IB, NCI
	D. H. Lynch	Investigator	IB, NCI
	A. Finnegan	Senior Staff Fellow	

COOPERATING UNITS *(if any)*

Centre d'immunologie, INSERM-CNRS de Marseille - Luminy
Marseille, France

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The responses of both cloned and heterogeneous T cell populations to mitogens and specific antigenic stimuli were found to require the participation of accessory or antigen presenting cells which express Ia (I region associated) determinants. Studies designed to analyze the functional importance of specific determinants on Ia molecules were carried out employing a battery of monoclonal anti I-E reagents specific for different epitopes on the same I-E product molecule. A series of T cell clones which recognized the same antigenic molecule in association with a given I-E molecule were studied for their susceptibility to inhibition by a panel of 15 different anti I-E monoclonal antibodies specific for determinants on the same I-E molecule. The results demonstrated that different T cell clones appear to recognize antigen in association with distinct determinants or conformations on the same I-E molecule. The responses of T cell clones to the mitogen Mycoplasma arthritidis supernatant (MAS) were analyzed. It was determined that approximately 15% of the clones tested responded to MAS when presented in the context of I-E bearing antigen presenting cells, and that this reactivity was independent of the primary specificity or MHC restriciton of those clones to conventional antigens. The ability of different Ia expressing populations to present to cloned T cells was evaluated. All of the clones tested were able to respond to adherent cell-containing irradiated stimulating cells or to mitomycin treated purified resting B cell populations. In contrast, only a subpopulation of cloned T cells was responsive to LPS activated B cell blasts, in spite of the enhanced Ia expression by these B cell blasts. These findings indicate that the signals required for T cell activation may vary among T cell clones, and that distinct Ia bearing antigen presenting cells may be competent for presentation to different T cells. Additional studies employing cloned Ia+ B cell lymphomas and hybridomas also indicate a heterogeneity among T cells in their responsiveness to these populations.

Project Description

Objectives: Ia antigens are serologically demonstratable cell surface determinants which appear to play important roles in cell-cell interactions. The objective of this study is to investigate the role these Ia determinants play in cellular interactions involved in T cell-mediated or T cell-dependent responses. The T cell proliferative response to mitogens and to soluble antigens, and T cell-dependent in vitro antibody responses are being studied. Studies employing monoclonal antibodies and cloned T cell populations have been directed at defining the functional importance of different determinants on the same Ia molecules.

Methods Employed: Cloned T cell populations have been generated by in vivo priming with soluble antigens, in vitro re-stimulation, and limiting dilution. These clones include I-A or I-E restricted T cells specific for antigens including KLH, GLPhe, and GLleu. Monoclonal anti-Ia antibodies have been employed including a panel of 15 anti-I-E^k antibodies specific for different domains on the I-E^k molecule as defined by competitive binding. Antigen-presenting cells have included both heterogeneous spleen cell populations and cloned B cell lines.

Major Findings: Cloned T cells specific for the antigens GLPhe, GLleu, KLH, and cytochrome C were generated and were shown to be both antigen-specific and under the control of complementing immune response genes in the I-A and I-E subregions. The proliferative responses of these cloned T cells were selectively inhibited by anti-I-E monoclonal antibodies. More specifically, it was shown that individual clones had different patterns of susceptibility to inhibition by the panel of anti-I-E antibodies. These findings suggest that different T cell clones may recognize antigen in association with different epitopes on the same Ia molecule. The supernatant of Mycoplasma arthritidis has previously been shown to be mitogenic for T cell populations. A panel of cloned T cells of varying MHC restriction and antigen specificity was employed to study reactivity to MAS. When T cell depleted populations of antigen presenting cells were employed, it was found that approximately 15% of T cell clones were reactive to MAS and were reactive only when I-E expressing antigen presenting populations were employed. The functional role of I-E products in these responses was confirmed by the inhibition of cloned T cell response by anti I-E monoclonal antibodies. Responsiveness to MAS + I-E product was independent of the primary antigen specificity or MHC restriction specificity of the responding cloned T cells.

In order to determine the requirements for T cell activation by Ia^+ antigen presenting cells, different Ia^+ populations were compared for their ability to trigger proliferative responses in cloned T cells. All of the T cell populations tested were Ia restricted in their responses, and all T cells responded to irradiated adherent cell-containing stimulating populations or to mitomycin C treated purified resting B cell populations. In contrast, some but not other cloned T cells were responsive to LPS activated B cell blasts of appropriate Ia phenotype. In addition, the use of monoclonal B cell lymphoma and hybridoma populations revealed a heterogeneity among T cells, with some but not other clones responsive to these B cell populations. These findings indicate that there is a heterogeneity in T cell activation requirements, such

that some but not other Ia expressing populations are competent to activate a given cloned T cell.

The role of antigen processing in presentation to T cells was studied through the use of chloroquine treatment of antigen presenting populations. This treatment, which serves to inhibit lysosomal processing, interfered with the ability of presenting populations to process and/or present soluble antigens to cloned T cells. In contrast, the responses of the same or different cloned T cells to self (autoreactive) or allogeneic (alloreactive) Ia determinants was was not influenced by treatment with low concentrations of chloroquine. These findings indicate that the in vitro antigen processing requirements for foreign soluble antigens are distinct from the requirements for presentation of intrinsic cell surface Ia determinants.

Significance to Biomedical Research and the Program of the Institute: I region gene products play a significant role in regulation of immune responses. A basic understanding of the expression, and perhaps the functional role of one class of I region gene products should provide insight into the mechanisms of antigen presentation and T cell recognition in immune responses. The findings to date suggest that discrete sites on Ia molecules are differentially important in antigen recognition by different T cell clones. This may reflect a heterogeneity in the sites on Ia molecules available for interacting with specific antigens and/or a heterogeneity in the Ia sites which can be recognized by T cells. The finding that individual T cell clones differ in their responsiveness to Ia^+ B cell populations has substantial significance in understanding the interaction between T cells and B cells. The possibility that, for example, certain T helper populations are capable of interacting with resting, but not activated, B cells would have important implications for the understanding of T cell regulation in B cell responses.

Proposed Course of Project: Cloned lines of antigen-presenting cells have been generated which express I-A and I-E products of several different haplotypes. These lines and variants derived from them will be utilized to further compare the activation and recognition requirements of different cloned T cell populations.

Publications:

Needleman, B. W., Pierres, M., Devaux, C. A., Dwyer, P. N., Finnegan, A., Sachs, D. H. and Hodes, R. J.: 1984. An analysis of functional T cell recognition sites on I-E molecules. J. Immunol. 133:589-596.

Finnegan, A., Needleman, B. W., and Hodes, R. J.: 1985. Antigen processing requirements for T cell activation: Differential requirements for presentation of soluble conventional antigen vs cell surface MHC determinants. J. Immunol. 134:2960-2965.

Finnegan, A., Smith, M. A., and Hodes, R. J.: Cloned T cell responses to macrophages and B lymphoblasts. In Streilein, J. W., Ahmad, S. B., Blomberg, B., and Voellmy, R. W. (Eds.): Advances in Gene Technology: Molecular Biology of the Immune System, Cambridge, Cambridge University Press, 1985, pp. 155-156.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05085-07 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Syngeneic Tumor Immunity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: G. M. Shearer Senior Investigator IB, NCI

COOPERATING UNITS *(if any)*

J. Hochman, Department of Biology, Hebrew University, Jerusalem, Israel

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3		0.3

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mice of the BALB/c strain injected with a line of the syngeneic T cell lymphoma S-49 which grows in suspension accept the tumor and die within two weeks. BALB/c mice injected with a plastic adherent (7.3) line of the same tumor are not killed. Furthermore, mice injected first with the 7.3 line and subsequently challenged with TAS are protected from the syngeneic tumor. Spleen cells from mice protected with 7.3 and challenged with TAS can be adoptively transferred to naive BALB/c mice which then protects these recipients. The 7.3 cell line appears to produce a factor in culture that stimulates growth of B lymphocytes. This may be a factor involved in immune protection against the metastatic TAS line.

No progress was made in this project during past year.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: The aim of this project is to study the mechanistic aspects of acquired immunity of mice to syngeneic T cell tumors. The model chosen for this study is that of the S-49 T cell Lymphoma of BALB/c origin, for which tumorogenic (TAS) and immunogenic (7.3) lines have been developed. Since injection of BALB/c mice with 7.3 protects against development of the tumor when challenged with TAS, analysis of the mechanism(s) of protection is planned. A long-term objective of the project is to determine if immunogeneic and tumorogenic lines of other murine T cell tumors can be developed.

Methods Employed: BALB/c mice were injected ip with $10\text{-}30x10^6$ TAS or 7.3 tumor cells. Mice protected by injection with 7.3 were challenged with $10\text{-}30x10^6$ TAS cells ip. Sera and splenic lymphocytes from the injected mice were adoptively transferred to other BALB/c mice, which were subsequently challenged with TAS.

Major Findings: In confirmation of Hochman's original observations, we found that: (a) tumor growth followed by death of the animals was observed in BALB/c mice injected with TAS but not in the mice injected with 7.3; and (b) injection of mice with 7.3 followed 1 to 9 months later with TAS did not result in tumor growth nor in death of the mice.

Significance to Biomedical Research and the Program of the Institute: The model outlined above raises the possibility of an approach for developing immunity against autologous T cell lymphomas - possibly by their in vitro growth properties (suspension-growing TAS which results in tumor growth in vivo vs plastic-adherent 7.3 which protects against in vivo tumor growth).

Proposed Course of Project: More detailed studies of the antibodies produced as a result of injection of these cells will be performed to determine whether any differences can be detected in the antibodies resulting from TAS vs 7.3 injection. For example, these antibodies could be of identical or somewhat different specificities and/or affinities. If the antibodies are identical it may be that both cell lines are equally immunogenic, but that only TAS is metastatic in vivo. It is also possible that the B cell stimulating factor(s) produced by T.3 enhance antibody production to antigenic determinants shared by T.3 and TAS. We plan to compare the levels of antibodies produced against possible shared determinants by the two lines.

Adoptive transfers of spleen cells and separated T and B lymphocytes from 7.3-injected, protected mice will be made into untreated BALB/c mice. These mice will then be challenged with TAS to determine if adoptive transfer of the cells confer protection against the tumorgenic line. If so, then the cell type (T or B) responsible for protection can be identified. At this point attempts will be made to clone the "protective" cells.

The demonstration of the above-outlined protective effect is of limited potential value if the phenomenon can be demonstrated for only one tumor. Therefore, one long-term goal of this project is to attempt to develop this

system using other lymphoid tumors. Some 40 radiation-induced T cell lymphomas are available to this laboratory for such studies.

The B cell stimulating factor produced in culture by the 7.3 cell line will be investigated for the properties responsible for stimulation of B lymphocytes.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05086-07 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immune Response Gene Regulation of the Immune Response In Vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Hodes	Chief, Immunotherapy Section	IB, NCI
Others:	D. H. Sachs	Chief, Transp. Biol. Sec.	IB, NCI
	A. Finnegan	Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Usa standard unraduced type. Do not exceed the space provided.)*

The cellular expression of immune response (Ir) gene function was studied in both primary and secondary in vitro antibody responses to the TNP conjugates of (T,G)-A--L and (H,G)-A--L. The function of accessory cells in responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L is under the control of genes which also map to I-A. In contrast, the expression of Ir gene function by B cells is related to the B cell activation pathway; Ir gene function is expressed by B cells activated under conditions involving MHC-restricted T-B interaction. In vitro augmented primary and secondary responses to TNP-nuclease (TNP-NASE) have also been established and documented to be under the control of H-2 linked Ir gene(s) mapping to the I-B subregion. For these responses, accessory cell function was shown to be under Ir gene control. Recent data employing monoclonal anti-Ia reagents have suggested that genes in the I-A subregion may also be involved in regulating responses to TNP-NASE. In order to further analyze the genetic regulation of T cell responses to NASE, a series of cloned lines were generated in BALB/c ($H\text{-}2^d$) as well as ($H\text{-}2^b$ x $H\text{-}2^a$)F_1 T cells. Individual BALB/c clones were restricted to recognizing NASE in the context of either $I\text{-}A^d$ or I-E products. Individual F_1 clones were specific for NASE in association with either $H\text{-}2^a$ or $H\text{-}2^b$ antigen-presenting cells and subregion mapping studies are currently in progress.

The antigen fine specificity of cloned NASE-specific T cells is also being probed through the use of mutant NASE molecules, synthetic peptides corresponding to segments of NASE, and monoclonal antibodies specific for different determinents on the NASE molecule.

Project Description

Objectives: The major objective of this project is to investigate the mechanism of genetic regulation of antibody responses. Initial studies identified the cellular level of Ir gene expression for the in vitro responses to TNP-(T,G)-A--L, TNP-(H,G)-A--L and TNP-NASE. Subsequent studies assessed the Ir gene regulation of activation of defined B cell subpopulations. In addition, since evidence for the existence of the I-B subregion is limited, an attempt will be made to study further regulatory phenomena mapped to I-B. The possibilities will also be tested that responses to different determinants on the same antigen molecule are under differential Ir control.

Methods Employed: The methods employed have been described in detail. See project No. Z01 CB 05064-05 I.

Major Findings: The in vitro primary and secondary antibody responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L are under strict H-2 linked gene control by genes mapping to the I-A subregion. When B cells are activated by a pathway requiring H-2 restricted T-B interaction, B cell function is found to be under Ir gene control. In contrast, in responses requiring no restricted T-B interaction, B cell function was not under Ir gene control.

In vitro TNP-specific responses to TNP-conjugated Staphylococcal nuclease (TNP-NASE) were generated by spleen cells from NASE-primed mice. These responses were T-cell and accessory cell-dependent, and under H-2-linked Ir gene control, with strains of the $H\text{-}2^a$ haplotype being responders and $H\text{-}2^b$ strains nonresponders. Ir gene control mapped to I-B and was not explained by complementing genes in I-A and I-E/C. Cell fractionation experiments have shown that accessory cell function is under Ir gene control for the response to TNP-NASE.

Monoclonal T_H cell populations specific for NASE have been generated in BALB/c ($H\text{-}2^d$) and (B10xB10.A)F_1 ($H\text{-}2^b \times H\text{-}2^a$) genotypes. Of the BALB/c clones tested, individual clones show MHC restriction for either I-A or I-E products. Studies employing fragments of the NASE molecule show that these BALB/c clones, all of which are responsive to the native NASE molecule, respond either to the 1-126 fragment or to no fragments at all. Individual (B10 x B10.A)F_1 clones respond to native NASE in association with either $H\text{-}2^a$ or $H\text{-}2^b$ antigen-presenting cells, and were shown by the use of intra-H-2 recombinants to be restricted to either $I\text{-}A^b$ or $I\text{-}E^k$. Mutant NASE variants as well as synthetic peptides corresponding to NASE sequences have recently been acquired and are being screened for the ability to stimulate NASE-specific clones.

Significance to Biomedical Research and the Program of the Institute: Genetic control of immune responses has been demonstrated in widely studied systems, including those responses to biologically "natural" antigens including allergens, viral determinants, and tumor antigens. In order to understand the mechanism of differential reactivity and susceptibility to these natural stimuli, the mechanism of Ir gene regulation of responses to defined stimuli may provide informative insights.

Proposed Course of Project: Cloned T cell populations will be analyzed for MHC restriction and antigen fine specificity. Conventional and monoclonal anti-Ia antibodies and anti-NASE antibodies will be used to probe for the I region products which function in Ir gene expression.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05088-07 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charactars or less. Title must fit on one line between the borders.)*

Effects of Graft Vs. Host Reactions on Cell-Mediated Immunity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. M. Shearer	Senior Investigator	IB, NCI
Others:	M. Moser	Fogarty Fellow	IB, NCI
	I. Iwasaki	Fogarty Fellow	IB, NCI
	F. Hakim	Staff Fellow	IB, NCI
	T. Malek	Staff Fellow	LI, NIAID

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The intravenous injection of F_1 hybrid mice with parental T cells result in a loss in the ability of the F_1 mice to generate T-cell mediated immune responses responses in vitro to graft-versus-host immune deficiency (GVHID). Recognition of host class II MHC antigens by donor cells is required to initiate GVHID. Recognition of host class I MHC antigens doesn't induce GVHID. Recognition of class II only results in loss of self + X responses but not of allogeneic responses; recognition of class I and II results in loss of self + X and allogeneic responses. Induction of GVHID by class I and II recognition requires both $L3T4^+$ and $Lyt2^+$ cells; induction of GVHID by class II only recognition requires only $L3T4^+$ parental T cells.

GVHID is accompanied by loss of ability to produce IL 2 and in loss of expression of IL 2 receptors. This IL 2 loss was observed in both class I and II and class II only GVHID. Recovery of immune function from GVHID was preceeded by recovery of IL 2 receptor expression and IL 2 production/

GVHID was used to abrogate natural resistance to bone marrow grafts in F_1 hybrid mice.

GPO 914-918

Project Description

Objectives: The purpose of this project is to investigate the phenomenon of immunosuppression induced by or associated with a graft vs. host reaction, the immunogenetics associated with the resistance in some strain combinations of the graft vs. host associated suppression, and to attempt to establish whether this immunosuppression is correlated with autoimmune states and/or the development of immune deficiency states following a graft vs. host reactions.

Methods Employed: F_1 hybrid mice of various strains were injected intravenously with from 1 to 40×10^6 F_1, parental, or allogeneic spleen cells. Inbred mouse strains were also injected with allogeneic cells. At various times after injection, the spleens of the injected F_1 mice were sensitized in vitro against: (a) parental or F_1 syngeneic cells modified with TNBS; or (b) allogeneic spleen cells. The effector cell actively generated 5 days later was tested on the appropriate ^{51}Cr-labelled target cells. F_1 mice were immunized to H-2 alloantigens or H-1 antigens prior to or at the same time that the F_1 mice were inoculated with parental cells. Chimera GVH mice were prepared by injecting F_1 mice with parental spleen cells, followed by irradiation and reconstitution with F_1 bone marrow cells. IL 2 production was measured by culturing spleen cells with ConA, harvesting the supernatants, and assaying the IL 2 activity on the HT2 cell line. IL 2 receptors were investigated using FMF by culturing spleen cells with the anti-IL 2 receptor monoclonal antibodies 7D4 and 3C7.

Major Findings: F_1 hybrid mice on the C57BL/10 genetic background injected intravenously with viable parental spleen cells lost their ability to respond by in vitro generated cytotoxic reactions to TNP-self and alloantigens. The induction of immune deficiency requires the recognition by parental T cells of allogeneic class II MHC determinants expressed by the F_1. GVHID induced by parent anti-host class II only results in loss of self + X responses, but not in loss of allogeneic responses, whereas class I + II-induced GVHID resulted in loss of self + X and allogeneic responses. The induction of class II only GVHID required only $L3T4^+$ parental cells, whereas induction of class I + II GVHID required $L3T4^+$ and $Lyt2^+$ cells.

Lymphocytes from mice that are immunosuppressed by GVHID produce reduced amounts of IL 2 and lose their IL 2 receptors. These results are in keeping with the observations that the addition of exogenous IL 2 to cultures of lymphocytes from GVH-suppressed mice restores immune function. This loss of IL 2 and IL 2 receptors is observed irrespective of whether GVHID was induced by class I + II or class II recognition. This suggested that allogeneic responses can occur in the absence of detectable IL 2 receptors. IL 2 receptor expression and IL 2 production began to reappear 8 weeks after GVHID induction and recovery of immune funciton began to reappear at 12 weeks. Active suppression of IL 2 reception was demonstrated by mixing spleen cells from normal and GVH mice.

Protection against GVH-suppression could also be induced by immunizing the F_1 mice to H-2 alloantigens prior to induction of suppression. This protection appeared to be antigen-specific in some but not all cases. Such immunization prevented the loss of GVH-induced IL 2 receptors.

Several parallels have now been established between AIDS in homosexual men and GVH-associated immunosuppression in our mouse model, which makes it an interesting model for studying induced immune deficiency. The most striking parallel observed is that both in class II GVHID and in the early stages of AIDS, self + X responses were lost whereas MHC allogeneic responses remained intact or were elevated. GVHID has been used to abrogate natural resistance to parental bone marrow grafts in lethally irradiated F_1 mice. This abrogation requires T cells in the parental inoculum, can be induced within 7 days, and is not necessarily associated with GVH induced loss of CTL potential. Although class I recognition alone does not result in GVHID, parental recognition of class I + minor H or mls determinants does result in GVHID. This raises the possibility that recognition of class II alloantigens can be replaced with recognition of self + X class II that will then lead to GVHID.

Significance to Biomedical Research and the Program of the Institute: The graft vs. host (GVH) reaction and possibly Hh-type reactions are important complicating factors which affect the success of hemopoietic transplantation. Since several recent cases of human marrow transplantation involving treatment of marrow with anti T cell reagents have resulted in failure to engraft, it may be that a chronic GVH is necessary to promote engraftment. Our GVH abrogation of natural resistance to marrow grafts may be relevant for this. Furthermore, persisting GVH reactions may be associated with autoimmune disease and the development of tumors. The observations: (a) that GVH reactions can be elicited with low numbers of lymphocytes in immunocompetent adult mice (previous reports have been limited to the demonstration of GVH in neonates or immunosuppressed animals); (b) that these GVH reactions lead to severely impaired T-cells immune functions; and (c) that such GVH reactions can be overcome by host resistance mechanisms are potentially of fundamental relevance in: (1) understanding the possible complications resulting from hemopoietic grafting; (2) investigating the significance of a GVH-induced suppressed immune system in the development of autoimmune and neoplastic disease; and (3) understanding natural resistance systems as they may be relevant in surveillance against disease and neoplasms. It is possible that our murine model has genetic and mechanistic relevance for SCID. It is also possible that GVH-associated immunosuppression may be a cofactor in the development of AIDS among homosexual men. Etiologic considerations of this type are consistent with the Program of the Institute.

Proposed Course of Project: We shall continue to investigate many aspects of the phenomenon including: (a) the genetics of the F_1 and parental cells involved; (b) the determinants recognized on the F_1 cells; (c) the mechanistic aspects of both the GVH and the suspected Hh component involved; (d) other immune functions which may be impaired including antibody production, delayed hypersensitivity, skin graft rejection, T-cell proliferative responses, and natural killer cell activity; (e) the long-term effects of the GVH including survival and the development of autoimmune disease and tumors; (f) which combinations of allogeneic cells and hosts can lead to GVH-associated immunosuppression; (g) analysis of different populations of cells involved in the induction of and protection by antibody against GVH-associated immunosuppression; (h) whether GVH-associated suppression is a useful model for

induced immune deficiency states; and (i) the interleukin defects associated with this suppression.

Publications:

Lang, P., Miller, M. W., and Shearer, G. M.: 1985. Failure of bone marrow cells to reconstitute T cell immunity in graft-vs.-host mice. J. Immunol. 134:2050-2052.

Joseph, L. J., Iwasaki, T., Malek, T., and Shearer, G. M.: 1985. Interleukin 2 dysfunction in mice undergoing a graft-versus-host reaction. J. Immunol., in press.

Shearer, G. M., Moser, M., Iwasaki, T.: 1985. Graft-versus-host-induced T cell immunodeficiency in mice. Immunol. Rev., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05099-05 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Synergistic Effects of Murine Cytomegalovirus and Graft-versus-host Reaction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. M. Shearer	Senior Investigator	IB, NCI
Others:	J. Titus	Chemist	IB, NCI
	C. Via	Medical Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

J. D. Shanley, University of Connecticut, Hartfort, CT
J. E. Grundy, Royal Free Hospital, London, U.K.

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mice injected with sublethal doses murine cytomegalovirus (MCMV) exhibit rapid and dramatic changes in their ability to generate in vitro cytotoxic T lymphocyte responses to hapten-self and to alloantigens, and to produce IL 2 and express IL 2 receptors. Within three days after intraperitoneal injection of MCMV, the CTL responses to hapten-self and alloantigens are abrogated or severely reduced. This is followed by rapid recovery to a normal level of CTL potential. Injection of F_1 hybrid mice with either MCMV or parental spleen cells (graft-versus-host reaction - GVHR) resulted in rapid and severe immunosuppression. Inoculation of either the virus or parental cells were selected so that they would be below the threshold for severe immunosuppression. However, when these two inocula were combined, severe immunosuppression was observed. Furthermore, injection of F_1 mice with parental lymphocytes that recognize only class I MHC determinants does not result in immune suppression. However, a combination of class I recognition and MCMV infection results in profound immune suppression. Infection of host mice with MCMV prior to induction of GVH resulted in augmented immune suppression, whereas infection of donor mice with MCMV before induction of GVH resulted in reduced immune suppression. These studies permit the investigation of the immunosuppression of MCMV infection and the possibility consequences of CMV infection coupled with a GVHR.

Project Description

Objectives: The purpose of this project is to investigate the immunological and genetic effects of MCMV infection and of MCMV infection plus a GVHR and for HVGR on (a) acquired T cell immunity to hapten-self antigens and alloantigens; and (b) on natural resistance to MCMV infection and the GVHR. Since certain mouse strains are relatively resistant to MCMV and to parental T cell-induced GVHR, it will also be the purpose of this study to investigate the role of H-2-linked and non-H-2-linked genetic effects of resistance and susceptibility to MCMV, to GVHR and to a combination of MCMV and allogeneic cells.

Methods Employed: Sublethal doses of MCMV (prepared from salivary glands of infected mice) were injected intraperitoneally into various inbred and F_1 hybrid mouse strains. Also F_1 mice were injected intraveneously with known concentrations of parental spleen cells, and F_1 mice were also injected with MCMV plus parental cells. The T cell immune potentials of injected and control mice were tested by in vitro sensitization to hapten-self and alloantigens, and the CTL activity was determined 5 days later using the ^{51}Cr-release assay. Changes in IL 2 receptor expression was determined by Flow Microfluorometry, using the 7D4 murine anti-IL 2 receptor monoclonal reagent. IL 2 production was measured on the HT2 cell line.

Major Findings: The injection of sublethal doses of MCMV resulted in rapid suppression of CTL potential to both hapten-self and allogeneic antigens (within 3 days). This was followed by recovery (by around 7 days), and augmented CTL activity as detected by the hapten-self and not by the allogeneic CTL systems (days 9-13). The injection of F_1 mice with doses of MCMV plus parental spleen cells each of which alone did not drastically reduce CTL potential, resulted in synergistic effect which abrogated CTL potential. This synergistic effect of GVHR and MCMV was most dramatically demonstrated using parent into F_1 in which only class I MHC antigens are recognized. Under such conditions GVH-associated immune suppression is never observed. However, when administered in conjunction with MCMV, severe suppression was observed.

The MCMV-GVHR synergy model was further analyzed by infecting GVH donor or host with MCMV prior to induction of GVHR. The results indicated that immune suppression: a) was more severe when host was infected prior to GVH-induction; and b) was less severe when donor was infected prior to GVH-induction.

Infection with MCMV results in depressed IL 2 production and in some loss of IL 2 receptors. The synergistic effect of sub-threshold doses of MCMV and GVHR were clearly demonstrated by drastic reduction in IL 2 production and IL 2 receptor expression.

Significance to Biomedical Research and the Program of the Institute: Cytomegalovirus infection is one of the major problems currently facing human bone marrow transplantation, and may become critical in patients undergoing a chronic GVHR. An understanding of the genetic and mechanistic paramaters involved in resistance and susceptibility to CMV in the murine model, the immunosuppression associated with CMV infection, and the possible synergistic

effects of CMV infection and chronic GVH should be valuable for both basic and clinical purposes.

Proposed Course of Project: We plan to attempt to introduce immune suppression in allogeneic combinations of mice that differ at minor H loci only using the combinaiton of MCMV infeciton and minor H locus recognition. We also plan to start a marrow transplant model in which mice will be lethally irradiated and grafted with allogeneic marrow (major or minor H locus differences), with and without MCMV infeciton. We shall monitor immune function in the chimeras as well as look for signs of GVH disease. Attempts will also be made to develop (with Dr. H. C. Morse, NIAID) the MCMV as a helper virus for a murine retrovirus that induces AIDS-like lesions in mice.

Publications:

Grundy, J. E. and Shearer, G. M.: 1984. The effect of cytomegalovirus infection on the host response to foreign and hapten modified histocompatibility antigens. Transplantation 37:484.

Grundy, J. E., Shanley J. D., and Shearer, G. M.: 1985. Augmentation of graft-versus-host reaction by cytomegalovirus infection resulting in interstitial pneumonitis. Transplantation, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - **PUBLIC HEALTH SERVICE**

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05100-05 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Role of HLA Genes in Human Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Shaw	Senior Investigator	IB, NCI
Others:	R. Hoffman	Medical Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

D. Glass, Brigham and Women's Hospital, Boston, MA; J. Hansen, Director, Histocompatibility Laboratory, Seattle, WA

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

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☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have defined an HLA locus (DP) which maps centromeric to the other known genes of the HLA complex. We are analyzing the importance of the genetic region marked by this gene in human disease. Family and population studies have been performed to analyze genetic control of pauciarticular juvenile rheumatoid arthritis. The data indicate that: 1) DPw2 is a new marker for susceptibility to pauciarticular JRA; 2) an individual's susceptibility to disease is influenced by at least two HLA-linked genes: one that is associated with DPw2 and another (or others) that is associated with DR5 or DRw8; 3) these susceptibility genes confer risk for the disease whether present on the same haplotype or opposite haplotypes. Studies are continuing on the frequency and importance of DP-mismatch on bone marrow transplantation between otherwise HLA-identical siblings. One DP-DR recombination has been identified in studies of 18 donor-recipient pairs. This finding increases confidence in the estimates of recombination frequency between DR and DP; together with further studies, this data will allow interpretation of the role of DP-mismatch in bone marrow transplantation.

PHS 6040 (Rev. 1/84) GPO 914-916

Project Description

Objectives: Previous studies from many laboratories have demonstrated that there are associations between many specific diseases and particular HLA antigens. For virtually all of these associations it is not known: 1) exactly what gene product is involved in the disease pathogenesis (i.e., whether the HLA gene product identified is involved in the disease or some unknown allele with which it is in linkage disequilibrium); and 2) what the mechanism is for the association. The extraordinary number of HLA associated diseases and the overall importance of the HLA region in immune responses suggest that an understanding of these associations may be of rather general relevance. In addition, the HLA region is known to be crucial in an "iatrogenic disease", transplantation rejection. As we develop new markers of the HLA region (project Z01CB05101 I), we expect they will be useful in helping us to map more precisely the gene products involved in disease associations and transplantation rejection.

Methods Employed: Patients are selected by diagnostic criteria relevant to the particular disease. Patients peripheral blood lymphocytes are "typed" for DP antigen expression by primed lymphocyte typing techniques using cellular reagents developed in this lab under project Z01CB05101 I; they are also serotyped for other HLA antigens under contract N01CB33935.

Major Findings: Glass and coworkers have demonstrated that a subset of patients with juvenile onset rheumatoid arthritis (JRA) have a markedly increased frequency of the DR5 (and DRw8) antigens as well as of HLA-B and-C antigens found in linkage disequilibrium with them. Studies of the frequency of the DP alleles in this disease were initiated to determine if the DP locus were included within the "extended" HLA haplotype which was associated with susceptibility to the disease. Cells from 30 patients with JRA were DP-typed by primed lymphocyte typing with standard bulk reagents and with two 'DPw2-specific' T cell clones. There was an increased frequency of the DPw2 antigen (66%) compared with normal controls (31%). However, the increase in DPw2 could not readily be accounted for by linkage disequilibrium between DR5 and DPw2 because: 1) DPw2 was found at the same frequency in donors who lacked DR5 as those who had DR5; and 2) linkage disequilibrium has not been observed between DR5 and DPw2 in normal populations. This suggested that the risk associated with DPw2 might reflect the existence of an additional HLA-linked in strong linkage disequilibrium with DPw2. Family studies defined the chromosomal relationship between DR5 or DRw8 and DPw2 in nine patients. In five the DPw2 antigen was encoded on the chromosome opposite to DR5 or DRw8; in three the antigen was on the same chromosome as DR5; and in 1 the DPw2 antigen was present on both chromosomes. These data indicate that: 1) DPw2 is a new marker for susceptibility to pauciarticular JRA; 2) an individual's susceptibility to disease is influenced by at least two HLA-linked genes: one that is associated with DPw2 and another (or others) that is associated with DR5 or DRw8; 3) these susceptibility genes confer risk for the disease whether present on the same haplotype or opposite haplotypes.

We had predicted that products of genes encoded in the HLA region around DP would function as histocompatibility barriers to allogeneic tissue transplantation. One specific context in which to test this prediction is in bone

marrow transplantation. Since DP differences may not result in "positive" MLC results, as many as 2-5% of MLC identical siblings may have undetected DR/DP recombinations. We have undertaken a collaborative study with J. Hansen (and the bone marrow transplantation team at Seattle) and have studied 18 "HLA-identical" siblings who are bone marrow transplantation donor-recipient pairs. Of these, only one pair are clearly DP-mismatched; family typing is in progress to confirm that this reflects DR/DP recombination. The DP typing was done "blind" and the clinical course of this pair has not yet been decoded. Additional samples are being collected prospectively on donor-recipient pairs to allow extension of these results. These studies have identified a probable additional DP-DR recombination which adds confidence to the estimates of recombination frequency and will allow functional studies of DP in members of the recombinant families. As further data are accumulated, they will allow evaluation of the effect of mismatch for DP on the outcome of transplantation.

Significance to Biomedical Research and the Program of the Institute: Many diseases are known to be HLA associated, including certain malignancies. Understanding of the role of HLA genes in the pathogenesis of these diseases might reasonably be expected to help in therapy and prevention of these diseases. Furthermore, if DP is important in transplantation, matching for DP would be expected to further improve the results of kidney and bone marrow transplantation.

Proposed Course of Project: The studies of JRA are strongly suggestive that DPw2 is a useful marker for disease susceptibility and studies will be extended with additional families who agree to participate. In the bone marrow transplantation study, we are assisting our Seattle collaborators in establishing DP-typing in their own laboratory, which will facilitate larger studies directed at: identifying DP-mismatched combinations, studying those families to define the recombinatorial event and its functional consequences, and correlating the mismatch with the clinical course of the bone marrow transplantation.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05101-05 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Definition of Human Histocompatibility Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Shaw	Senior Investigator	IB, NCI
Others:	M. Sanchez-Perez	Guest Worker	IB, NCI
	E. Long	Investigator	NIAID
	R. Sekaly	Investigator	NIAID

COOPERATING UNITS *(if any)*

V. Quaranta, Scripps Clinic, La Jolla, CA; R. DeMars, U. of Wisconsin, Madison, WI; J. Trowsdale, P. Austen, W. Bodmer, ICRF, London, England

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

Using T cell recognition (particularly cell-mediated cytotoxicity) we have continued to probe the complexities of the alloantigenic differences between normal human donors. In contrast to our previous studies trying to induce T cell responses against new determinants, we are analyzing, at the molecular level, the specificity of 10 'DPw2-specific' cytotoxic T lymphocyte (CTL) clones already generated. Analysis of their specificity on panels of MHC-loss mutant lymphoblastoid cell lines has been extended. These studies confirm the identification of CTL-defined differences between mutant cell lines which is not explained by differences in the DP gene, but rather appear to reflect mutation of another gene within HLA. Biochemical analysis suggests that the differences correlate with subtle differences on 2-dimensional gel electrophoresis in the Ia-related molecules precipitated by an anti-invariant chain antibody. We hypothesize that hybrid molecules (e.g., DP-beta + DR-alpha) may contribute to this recognition. Northern blot analysis is in progress to test the hypotheses that differences in expression of DR or DQ genes will correlate with these differences in recognition. Two other genetic approaches are being used to analyze the target cell requirements for killing by these CTL clones. First, mutant cell lines are being isolated by mutagenesis and negative selection with the CTL clones themselves (rather than with antisera). Of two mutants which have been derived and are being analyzed in detail, both have lost the capacity to be recognized by DPw2-specific CTL, but only one has lost serologic expression of DPw2. The genetic basis for these alterations are under investigation. Second, cells are being transfected with class II genes including those thought to encode DPw2. Our findings indicate that reconstitution of function as a target cell for DPw2-specific CTL requires more than expression of the DPw2 molecule. These additional requirements are being defined.

Project Description

Objectives: Genes of the HLA region are important in tissue transplantation, immune regulation and regulation of susceptibility to a variety of diseases. Consequently, it is crucial to define the gene products of this region and to determine the function of these gene products. There has been a worldwide effort to do so, primarily by serologic techniques. We believe that T cells may be the most sensitive probe for defining functionally important details of the HLA region, since T cells seem to be uniquely committed to recognizing gene products of this region. Therefore we expect refined approaches to cellular typing to allow definition of new HLA gene products. Once appropriate cellular reagents have been found to define these antigens, we will investigate the genetics of these markers, the tissue distribution of the markers, and function of the markers in cellular immune responses.

Methods Employed: Human PBL are obtained from donors by phlebotomy or batch leukapheresis; mononuclear cells are separated by density separation, and cryopreserved. Analysis of the serologically defined HLA markers on the donors cells is performed by microcytotoxicity testing under contract N01CB33935. Cells from carefully selected donors are sensitized in vitro in one way mixed lymphocyte culture, and generally the primed cells are restimulated after 10 days with the same stimulator to enhance weak responses. Primed cells are frozen in large batches, and thawed as necessary to provide standard "reagents". Proliferation of these cells in response to stimulator cells is measured by ^{3}H-thymidine incorporation. Cytotoxic activity is analyzed by short term ^{51}Cr release assays using as targets T lymphoblasts or lymphoblastoid B cell lines. For higher resolution analysis of the specificity and function of individual T cells, single cell cloning has been performed by limiting dilution and subsequent expansion in the presence of specific antigen and TCGF. Monoclonal antibody binding has been assayed by flow cytometry. Analysis of Ia molecules from cell lines has been performed by our collaborators by immunoprecipitation and 2D-gel electrophoresis using standard techniques. Mutant cell lines have been generated by inducing mutations of lymphoblastoid cell line 721 with ICR191, allowing 5-7 days for expression of the mutant phenotype, negative selection by incubation of those cells with cytotoxic T cell clones, then propagating at limiting dilution to clone the survivors. Cells have been transfected with DP genes by our collaborators using standard methods.

Major Findings: Last years annual report outlines surprising results of studies of the fine specificity of DPw2-specific CTL clones on panels of cells from unrelated donors and of HLA-mutant cell lines. Unexpected killing by clone 8.4 on some DPw2-negative target cells remains unexplained. Such anomalous killing can be partially blocked by anti-Ia antibody on some targets. Linkage studies in families have not provided conclusive results on HLA-linkage of the antigen recognized.

More extensive analysis has been conducted on the variations between clones in their ability to lyse some of the HLA-mutant cell lines which retain DPw2. The functional observation has been extended in several respects. 1) Cold target inhibition studies confirm the inability of clones such as 8.3 to 'recognize' DPw2-positive mutant LCL such as 721.139 but not a serologically indistinguish-

able mutant LCL 721.137. 2) The involvement of the DPw2 molecule in this killing was confirmed by blocking of CML with antibodies specific for DP-locus products (B7/21). 3) Additional mutant LCL pairs have been identified which are serologically indistinguishable but which differ in killing by CTL 8.3; some of these LCL pairs were derived with the mutagen ICR191 rather than with gamma-rays, and therefore may have changes in a single gene. 2D-gel electrophoresis has been used to try to identify differences between these cells with respect to total cell surface protein, or molecules immunoprecipitated with anti-Ia antibodies. No dramatic differences have been observed, but there is the suggestion of some differences in the spots immunoprecipitated with anti-invariant chain antibody. Molecular genetic analysis by Southern and Northern blotting with Ia probes is in progress. At present, our working hypothesis is that CTL clones like 8.3 may preferentially recognize a 'hybrid' molecule which includes a polymorphic chain encoded in the DP region and a relatively non-polymorphic chain encoded by a gene close to or identical with DR alpha or DR beta. If confirmed, this would be the first evidence for functional recognition of an Ia molecule comprised of chains from two different Ia "families".

Because the serologically derived mutants have demonstrated such complexity in the products recognized by T cells, we have extended the approach by deriving mutants which have been negatively selected by the CTL clones themselves, rather than by anti-HLA antibodies. This approach may identify structures which are relevant to the CTL but not to antibody. Methodology has been developed and four mutants have been derived by mutagenesis of LCL 721 with ICR191, culturing for 7d to allow expression of the mutant phenotype, then negative selection by two cycles of incubation with DPw2-specific CTL clone 8.9. The survivors were cloned by limiting dilution. They are not contaminating cells which outgrew, because extensive HLA-phenotyping confirms that they have the serological phenotype of parental cell. Two such mutants EM2 and EM6 have been subcloned and both are mutants since they cannot be killed by the clone 8.9 or any of the other DPw2-specific clones tested. They also cannot cold-target inhibit recognition by DPw2-specific CTL. However, they are not completely resistant to all lysis since they can be lysed in lectin dependent killing or by CTL of other specificity. They have been tested by flow cytometry for their ability to bind a wide variety of anti-Ia antibodies and antibodies against other immunologically relevant molecules. The only antibody which consistently demonstrated differences between them and the parental line is the DP-specific monoclonal ILR1. EM6 has lost its ability to bind ILR1, suggesting selective loss of expression of the DPw2 product. Surprisingly, EM2 has retained about 50% of the normal level of binding ILR1, indicating a different basis for its defect in recognition.

Studies of cell lines transfected with DP genes are complementing the foregoing approaches to analysis of antigens recognized by T cells. Murine L cells transfected with a DPw2 cosmid (alphal-betal-alpha2) express a 'DP' molecule identified by monoclonal antibodies but are not lysed by DPw2-specific CTL clones. Preliminary analysis of monkey COS cells transiently infected with DPw2 alpha and beta chains are also not lysed. Studies are in progress to determine whether failure of lysis relates to level of expression, to requirement of species-specific molecules other than DPw2 or to some other requirement for CTL recognition.

Significance to Biomedical Research and the Program of the Institute: Because genes of the HLA region are crucial in controlling immune responses, transplantation, and increasing the risk of a large variety of diseases, therapeutic intervention related to these phenomenon may depend on further understanding of the genes in this region. The DP gene defined already in this project promises to be a very informative one. The present studies clearly indicate that the way T cells 'see' DP is rather more complex than one might predict. Since such details of T cell recognition are essential to normal and pathophysiological host responses, our understanding of them may be essential to unravel the events involved in tumor recognition by T cells and in autoimmune responses.

Proposed Course of Project: We will continue to characterize the genetics, structure and function of products of the DP region of the HLA complex. Further mutant LCL will be generated by CTL selection and characterized at the functional, molecular, and genetic levels. Ia genes will be transfected into normal and HLA-deletion mutant cells to define the requirements for DPw2-specific CTL recognition.

Publications:

DeMars, R., Chang, C. W., Shaw, S., Reitnauer, P. J., and Sondel, P. M.: Homozygous deletions that simultaneously eliminate expressions of class I and class II antigens of EBV-transformed B-lymphoblastoid cells. I. Reduced proliferative responses of autologous and allogeneic T cells to mutant cells that have decreased expression of class II antigens. Hum. Immunol. 11:77-97, 1984.

Hartzman, R., Shaw, S., and Robbins, F. M.: Origin and expansion of SB-specific reagents characterized in the 9th International Workshop. In: Mayr, M., Albert, E., and Baur, M. P.: Histocompatibility Testing 1984. Berlin, Springer-Verlag, in press, 1984.

Nishimura, Y., Yasuda, N., Sasazuki, T., and Shaw, S.: The gene frequency of SB alleles in the Japanese population. Tissue Antigens 23:314-315, 1984.

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Sanchez-Perez, M., DeMars, R., and Shaw, S.: Mutant B cell lines selected by resistance to lysis by DPw2-specific cytotoxic T cell clones. In Streilein, J. W., Ahmad, F., Black, S., Blomberg, B., and Voellmy, R. W. (eds) Advances in gene technology: Molecular biology of the immune system. Cambridge University Press. p 285-286. 1985.

Shaw, S.: Non-immunological functions of MHC and MHC-linked genes. ASHI Quarterly. 9:12-12, 1985.

Shaw, S., and DeMars, R.: Binding specificity of anti-human Ia monoclonal antibodies analyzed with HLA-deletion mutant cell lines: patterns of binding to products of two different HLA haplotypes and of three subregions of a single haplotype. Disease Markers. 2:183-195, 1984.

Shaw, S., Goldstein, G., Springer, T. A., and Biddison, W. E.: Susceptibility of cytotoxic T lymphocyte (CTL) clones to inhibition by anti-T3 and anti-T4 (but not anti-LFA-1) monoclonal antibodies varies with the avidity of CTL-target interaction. J. Immunol. 134:3019-3026, 1985.

Shaw, S., Sanchez-Perez, M., and DeMars, R.: Analysis of DP-region products by T cells and monoclonal antibodies: blocking of DP-specific proliferation and cell-mediated cytotoxicity. In: Mayr, M., Albert, E., and Baur, M. P.: Histocompatibility Testing 1984. Berlin, Springer-Verlag. p 465-468, 1984.

Shaw, S., Ziegler, A., and DeMars, R.: Specificity of monoclonal antibodies directed against human and murine class II histocompatibility antigens as analyzed by binding to HLA-deletion mutant cell lines. Hum. Immunol. 12:191-212, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05103-04 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure and Function of Cytotoxic T Lymphocyte Granules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. A. Henkart	Senior Investigator	IB, NCI
Others:	T. Soares	Microbiologist	IB, NCI
	W. Munger	Staff Fellow	IB, NCI
	J. Bluestone	Laboratory Leader	IB, NCI
	C. Yue	Medical Staff Fellow	IB, NCI
	R. P. Blumenthal	Chief, Membrane Structure Sect.	LMMB, NCI

COOPERATING UNITS *(if any)*

S. A. Rosenberg	Chief, Surgery Branch	NCI

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cytoplasmic granules from cloned CTL were prepared by Percoll gradient centrifugation of homogenates prepared by nitrogen cavitation. In addition to the cytolysin these granules contain a DNAse activity capable of releasing 125I-UdR from nuclei. This nuclease activity is found in far smaller amounts in granules of non-cytotoxic lymphocytes, and the level of activity in cloned CTL is greater than that seen in LGL tumor cells. This enzyme is active at neutral pH, and was shown to cleave high molecular weight DNA. Thus it appears that the reports of rapid target cell DNA cleavage during CTL-mediated cytotoxicity can be accounted for by CTL granule DNAse without postulating endogenous DNAses in the target cell. In order to test the granule exocytosis model for lymphocyte toxicity on lymphocytes which are neither LGL nor classical CTL, cytoplasmic granules were prepared from mouse LAK cells, spleen lymphocytes cultures with recombinant IL-2 which develop cytotoxic activity. It was found that cytolytic activity in the granules appears on about day three of culture and continues to increase for the next 6 days. This pattern roughly follows LAK cytotoxic activity but begins later and does not plateau with time. IL-2 titration shows similar amounts of lymphokine are needed to generate granule lytic activity and LAK. The LAK cytolysin shows a similar spectrum of target cell killing and a similar dependence on calcium and rapid kinetics as do the cytolysins from LGL and CTL. The LAK cytolysin is also neutralized by anti-LGL granule antibodies. These results support the granule exocytosis model for LAK-mediated lysis.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: To characterize the cytoplasmic granules of cytotoxic T lymphocytes and other cytotoxic lymphocytes and ascertain their role in the lytic function.

Methods employed: Cloned alloreactive cytotoxic T cells are grown in in vitro culture with IL2 from EL4 and ConA stimulated spleen cell supernatents. They are cloned by limiting dilution methods and assayed for cytotoxicity by the ^{51}Cr release method. Granules were purified by the previously described Percoll gradient method. Cytolysin activity was measured routinely by serially diluting the material in PBS and adding an equal volume of SRBC suspended in BSS. Hemolysis was measured by hemoglobin release. ^{51}Cr release was also used with nucleated cell targets. LAK were generated by cultures of mouse spleen cells with recombinant IL-2

Major Findings: Cytoplasmic granules from cloned CTL were prepared by Percoll gradient centrifugation of homogenates prepared by nitrogen cavitation. In addition to the cytolysin these granules contain a DNAse activity capable of releasing 125I-UdR from nuclei. This nuclease activity is found in far smaller amounts in granules of non-cytotoxic lymphocytes, and the level of activity in cloned CTL is greater than that seen in LGL tumor cells. This enzyme is active at neutral pH, and was shown to cleave high molecular weight DNA. Thus it appears that the reports of rapid target cell DNA cleavage during CTL-mediated cytotoxicity can be accounted for by CTL GRANULE DNAse without postulating endogenous DNAses in the target cell. In order to test the granule exocytosis model for lymphocyte toxicity on lymphocytes which are neither LGL nor classical CTL, cytoplasmic granules were prepared from mouse LAK cells, spleen lymphocytes cultures with recombinant IL-2 which develop cytotoxic activity. It was found that cytolytic activity in the granules appears on about day three of culture and continues to increase for the next 6 days. This pattern roughly follows LAK cytotoxic activity but begins later and does not plateau with time. IL-2 titration shows similar amounts of lymphokine are needed to generate granule lytic activity and LAK. The LAK cytolysin shows a similar spectrum of target cell killing and a similar dependence on calcium and rapid kinetics as do the cytolysins from LGL and CTL. The LAK cytolysin is also neutralized by anti-LGL granule antibodies. These results support the granule exocytosis model for LAK-mediated lysis.

Significance to Biomedical Research and the Program of the Institute: It is clear that the cell-mediated immune system is responsible for many fundamental properties of the body's overall defense system against foreign organisms. This also appears to be true of the body's natural defenses against tumors. We are bringing a new means of analysis to bear on the cells which mediate these activities so that they can be understood on a molecular basis. This knowledge should be of great benefit in designing new therapeutic modalities.

Proposed Course of Project: Collaboration with Dr. Byoung Kwon has begun in order to identify the gene(s) in CTL which code for the granule cytolysin. Dr. Kwon has constructed a cDNA library in lambda gtll which is being screened using our anti-cytolysin antibodies. In order to better understand the granule exocytosis process in CTL, we will also attempt to develop

techniques for measuring the secretion event independent of the measurement of cytolytic activity. We will continue to make comparisons of CTL, LGL, and LAK granules.

Publications:

Henkart, P. A., Millard, P. J., Reynolds, C. W., Fredrickse, P., Bluestone, J. A., Blumenthal, R., and Henkart, M. P.: Cytolytic properties of cytoplasmic granules from LGL tumors and cytotoxic T lymphocytes. In Henkart, P., and Martz, E. (Eds.): Mechanisms in Cell Mediated Cytotoxicity II. New York, Plenum Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05104-04 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Detection and Analysis of H-2 Variant Cell Lines from Murine T Cell Lymphomas

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: G. M. Shearer Senior Investigator IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5		0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Various lines of the S-49 T cell lymphoma of BALB/c origin are being studied for the expression of H-2 antigens. Normal BALB/c lymphocytes express H-2K^d, H-2D^d, and H-2D^d antigens. We have found that the five lines of the S-49 lymphoma thusfar studied do not express all of these cell surface H-2 antigens. The patterns of expression of H-2 antigens using these cells as targets for: (a) antibody and complement; and (b) cytotoxic T lymphocytes (CTL) exhibit four different patterns of H-2^d expression in the five lines tested. This system may be of value for investigating regulation of expression of major histocompatibility complex (MHC) antigens, and raises the possibility of a relatively high rate of modulation of these antigens among tumor cell lines of the same origin.

No progress was made in this project during the past year.

Project Description

Objectives: The purpose of this project is to study the expression of H-2 antigens on different lines of a T cell lymphoma, to determine the mechanism(s) of differential expression of these antigens on the cell lines and to establish whether we can detect changes occurring in H-2 expression as we carry these lines in vitro and in vivo.

Methods Employed: The various tumor lines as well as "wild type" BALB/c cells were tested for expression of K^d, D^d and L^d antigens by CTL, monoclonal reagents plus complement, and monoclonal reagents using FACS. Internal labelling using "^{14}C-leucene for immunoprecipitation studies were performed to determine whether the lack of expression of these H-2 antigens are a problem of cell surface antigen expression or intra-cellular synthesis.

Major Findings: Of the five lines of S-49 thusfar examined, at least four different patterns of H-2^d antigen expression has been observed: the "7.3" and "TAS" lines (actually splits of the same line) $K^d(-)$, $D^d(+)$, $L^d(-)$; "100.0" $K^d(-)$, $D^d(-)$, $L^d(-)$; S-49.1 $K^d(+)$, $D^d(+)$, $L^d(-)$; S-49-Thy-$K^d(+)$, $D^d(-)$, $L^d(-)$. Using BALB/c anti-pool sera as well as BALB/c anti-pool CTL, both of which should detect antigens of any other known H-2 haplotype, no other H-2 antigens have been detected. This is compatible with the hypothesis that these cell lines are not mixed up or contaminated with any other murine tumor cell lines that would express other H-2 antigens. Due to projects of a higher priority, little progress has been made on this project during the past year.

Significance to Biomedical Research and the Program of the Institute: The modulation of major histocompatibility complex (MHC) antigens by tumor cells provides an interesting model for investigating expression of these antigens on cell surfaces as well as gene expression. Furthermore, since autologous tumor antigens appear to be recognized in association with syngeneic MHC antigens by T lymphocytes, the modulation of MHC antigens may be a mechanism by which tumors could escape rejection. The tumor lines we have identified that have "lost" certain H-2 antigens could represent lines which have been selected to grow in host mice by such an "escape" mechanism.

Proposed Course of Project: Biochemical analysis at the levels of cell surface expression and intracellular synthesis will be performed in order to understand this antigenic modulation. Other S-49 cell lines will be studied in an attempt to identify additional lines which exhibit differential H-2^d antigen expression. An extensive panel of anti-H-2^d monoclonal reagents will be employed to elucidate the fine specificity of the antigens expressed by these tumor cell lines. The tumor lines will be injected into syngeneic BALB/c and H-2 allogeneic mice in an attempt to recover cell lines that exhibit additional differences in H-2^d antigen expression. If a number of the above experiments are interesting it will be important to determine if the phenomenology is more general, and could be demonstrated for other murine T cell lympomas. Some 40 lines are available for these studies.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05106-04 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of the T Cell Alloreactive Repertoire

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Hodes	Chief, Immunotherapy Section	IB, NCI
Others:	R. Gress	Senior Investigator	IB, NCI
	D. H. Lynch	Investigator	IB, NCI
	B. Needleman	Medical Staff Fellow	IB, NCI
	J. Bluestone	Lab Leader	IB, NCI
	D. Singer	Senior Investigator	IB, NCI
	M. Pescovitz	Senior Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

Department of Pathology, University of Utah, Salt Lake City, UT

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
	1.0	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The alloreactive T cell repertoire has been analyzed for the influence of non-MHC determinants on alloreactivity. The responses of cloned T cells to Mls^a were analyzed in clones generated specifically to Mls^a stimulator cells as well as in clones specific for soluble antigens (antigen specific), foreign I region products (alloreactive), or syngeneic I products (autoreactive) which showed cross reactive recognition of Mls^a. It was demonstrated through the use of congenic strains, recombinant inbred strains, and monoclonal antibodies, that cloned T cell responses were specific for Mls and that this recognition was MHC restricted. The overall importance of non-MHC encoded determinants in alloreactive T cell recognition was surveyed by examining a panel of 70 antigen specific and/or autoreactive T cell clones for coincidental alloreactivity. Approximately 80% of the clones analyzed revealed one or more alloantigen specific cross reactivities. In several of these instances, specificity appeared to be for MHC products alone, without detectable influence of non-MHC genes. However, in the majority of instances, alloreactive specificity involved non-MHC as well as MHC gene products expressed by stimulating populations. These findings therefore suggest that the high frequency of alloreactive T cells observed in multiple experimental systems may reflect a frequent role of non-MHC as well as MHC encoded gene products.

The cytotoxic T cell repertoire specific for class I allogeneic and xenogeneic determinants was studied. Through the use of radiation bone marrow chimeras, it was demonstrated that responsiveness to K^b mutant determinants was the outcome of unique interactions between both T cell genotype and maturation environment. Through the use of a transgenic mouse model, in which porcine class I genes had been introduced into the germ line of murine cells, it was demonstrated that normal murine T cells expressed a cytotoxic T cell repertoire specific for xenogeneic class I determinants expressed on mouse cells. This repertoire was cross reactive with the alloreactive cytotoxic T cell repertoire.

Project Description

Objectives: T cell responses to Mls or MHC encoded alloantigens appear to be unique in the strength of these primary responses and in the correspondingly high precursor frequency of responding T lymphocytes. For both these conceptual reasons, and because of the importance of these loci in transplantation biology, a more complete understanding of response mechanisms to these determinants is both interesting and important.

Methods Employed: Mls-specific T cell clones were generated by in vitro culture and limiting dilution techniques, employing stimulator populations expressing Mls^a determinants. Other clones were generated in similar fashion by repeated stimulation with appropriate soluble antigens and presenting cells.

Studies of responses to the mutant K^b products employed a series of mutants including K^{bm1} and the K^{bm6}. Responses to these determinants were studied in assays of CML. Responding populations included normal spleen cell populations, radiation bone marrow chimeric spleen cell populations, and spleen cells from neonatally tolerized animals. Isolation of the swine class I MHC gene PD1 and its introduction by microinjection into fertilized mouse embryos are described in detail in Project No. Z01CB05124-01 I. Cytotoxic T cell responses specific for transgenic MHC products were evaluated through conventional techniques of in vitro sensitization and cytotoxic T cell assays.

Major Findings:

1) Cloned T cells were generated which are specific for Mls^a determinants, as demonstrated by strain distribution and by responses to recombinant inbred lines derived from (B6 x DBA/2)F_1 mice (heterozygous for Mls^a). The use of H-2 congenic and F_1 stimulating cells demonstrated that Mls^a was recognized by most clones in the context of $H\text{-}2^b$, $H\text{-}2^d$, or $H\text{-}2^k$, but not $H\text{-}2^q$ products. Individual clones utilized I-A or I-E products as restricting elements. A number of other T cell clones which were generated to be specific for soluble antigens (antigen-specific), foreign I region products (alloreactive), or syngeneic I products (autoreactive) also showed cross-reactive recognition of Mls^a in a similarly H-2-restricted fashion. The MHC restriction specificity of Mls^a recognition by cloned T cells was confirmed through the use of monoclonal anti I-A and I-E antibodies.

2) B6, B10, or B10.D2 mice, as well as B10.D2 --> B10 chimeric cells were all able to generate strong CML responses to K^b mutant bm1 and bm6 determinants. In contrast, however, B10 --> B10.D2 chimeric T cells had a markedly reduced response to bm1 and were entirely unresponsive to bm6, in spite of their normal alloreactivity to third party B10.BR. The same selective unresponsiveness to bm6 was observed in T cells from B6 --> bm1 chimeras, indicating that K region differences in maturation environment were sufficient to cause this effect. These findings demonstrate that the alloreactive T cell repertoire to these mutant K region determinants is not the product of T cell genotype alone (since either $H\text{-}2^b$ or $H\text{-}2^d$ T cells can respond) or of the T cell maturation environment alone (since T cells which have matured in either an $H\text{-}2^b$ or $H\text{-}2^d$ environment can be responsive). Rather, the alloreactive T cell repertoire appears to be the unique outcome of interaction between T cell genotype and maturation

environment, paralleling the phenomenon previously demonstrated for MHC-restricted T cell recognition of conventional antigens. Cell mixing experiments indicated that the restricted repertoire observed in these chimeric populations is a property of the cytotoxic T cell precursor population.

Normal B10 T cells were capable of generating cytotoxic T cell responses specific for transgenic B10 mice which expressed the serologically detectable product of the xenogeneic PD1 class I MHC gene. The specificity of this cytotoxicity was confirmed employing transfected L cell targets and by inhibition with alloantisera. In order to analyze the T cell repertoire specific for the transgenic gene product, cytotoxic T cells generated by a B10 antitransgenic sensitization were assayed for cross reactivity against a panel of allogeneic murine target cells. It was observed that there was variable cross reactive lysis on a panel of fully MHC allogeneic target cells. In contrast, a consistent and strikingly high degree of cross reactivity was seen against a number of K^b mutant target cells. These findings suggest that the expressed cytotoxic T cell repertoire for xenogeneic class I MHC products is cross reactive with allogeneic class I determinants and that this repertoire may bear a selective relationship to the K^b mutant specific repertoire.

Significance to Biomedical Research and the Program of the Institute: It remains a central question whether the mechanisms of immune repertoire generation are identical for conventional antigens and for MHC encoded antigens. In this respect, the results of the current studies have demonstrated that in several respects the strong T cell responses to both Mls and mutant K^b determinants parallel response mechanisms studied for MHC-restricted responses to conventional antigens. The most immediately apparent implications of such findings include their application to situations such as clinical bone marrow transplantation, in which complex influences can be anticipated upon the ultimate host response repertoire to transplantation as well as conventional antigenic challenge. Analysis of the T cell repertoire toward xenogeneic transplantation antigens will bear specific relevance for the facilitation of xenogeneic tissue or organ grafting.

Proposed Course of Research: Studies will be carried out to examine the incidences of Mls recognition by T cell clones of other primary specificities. Antibodies specific for T cell receptor structures will be utilized to determine whether the receptors used in recognition of Mls^a are the same or different from those used in recognition of other specificities. Further studies of the T cell repertoire specific for xenogeneic MHC determinants will be carried out employing T cell populations derived from bone marrow chimeras as well as cloned T cell populations.

Publications:

Lynch, D. H., Gress, R. E., Needleman, B. W., Rosenberg, S. A., and Hodes, R. J.: 1985. T cell responses to Mls determinants are restricted by cross-reactive MHC determinants. J. Immunol. 134:2071-2078.

Ryan, J. J., Gress, R. E., Hathcock, K. S. and Hodes, R. J.: 1984. Recognition and response to alloantigens in vivo. II. Priming with accessory cell-depleted donor allogeneic splenocytes: Induction of specific unresponsiveness to foreign

major histocompatibility complex determinants. J. Immunol. 133:2343-2345.

Lynch, D. H., Needleman, B. W., and Hodes, R. J.: T cell responses to Mls determinants are restricted by polymorphic determinants shared between I-A and I-E molecules. In Streilein, J. W., Ahmad, S. B., Blomberg, B., and Voellmy, R. W. (Eds.): Advances in Gene Technology: Molecular Biology of the Immune System, Cambridge, Cambridge University Press, 1985, pp. 239-240.

Frels, W. I., Bluestone, J. A., Hodes, R. J., Capecchi, M. R. and Singer, D. S.: 1985. Expression of a microinjected porcine class I MHC gene in transgeneic mice. Science 228:577-580.

Bluestone, J. A., Frels, W. I., Pescovitz, M. D., Singer, D. S., and Hodes, R. J.: 1985. T cell recognition of xenogeneic MHC in transgenic mice. Advances in Gene Technology: Molecular Biology of the Immune System, Vol. 2, pp 113-114.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05107-04 I

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
T Cell Responses to Minor Histocompatibility Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Rosenberg	Medical Staff Fellow	IB, NCI
Others:	A. Singer	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ability to generate cytotoxic T lymphocyte responses to minor H antigens offers a potent tool for the study of self-tolerance and self-recognition. Results obtained in this system have thus far demonstrated 1) that the self + X T cell repetoire is highly cross-reactive for allogeneic MHC determinents suggesting that the response to allogenic MHC antigens is comprised of multiple self + X specificities and 2) that self minor H determinents tolerize T cells only in association with self MHC determinents so that tolenance induction to non MHC self components is restricted by MHC encoded products. We are currently examining the role of antigen processing in the generation of the cytotoxic response to minor-H antigens. Results so far indicate that 1) Macrophages are requisite for minor H specific CTL generation in vitro. 2) The minor antigens need not be synthesized by the antigen presenting cell but can be acquired in vitro by macrophages and subsequently presented in an immunogenic fashion. 3) The generation of the CTL by non antigen bearing APC's is inhibitable by antibodies to the T4 molecule expressed by Ia-restricted T helper cells.

Project Description

Objective: These experiments will study the mechanism of the development of tolerance and reactivity to minor histocompatibility antigens.

Methods Employed: Normal and chimeric mice are primed with spleen cells which express allogeneic minor H antigens. After 2.5 weeks, recipient thymus or spleen cells are restimulated in vitro and the generation of cytotoxic T cells specific for allogeneic minor H antigens is assessed. Spleen cells from mice primed in vivo to minor H antigens were either left unseparated or were macrophage depleted. Depletion was accomplished via G10 Sephadex columns. Purified accessory cell populations (obtained via RAMB/complement treatment of whole spleen population) of either stimulator or responder phenotype were added and were both shown capable of restoring the response. The effects of MT4, L3T4 and anti IA antibodies are being assayed in an effort to establish the dependency or independence of the response on IA molecules. The effects of chloroquin, a drug capable of interfering with antigen processing are also being studied in an effort to delineate the role of processing.

Major Findings: 1) The MHC dependence of T cell tolerance induction to minor H antigens was investigated using parent --> F_1 radiation bone marrow chimeras of the form C3H.SW --> (B10xBR)F_1. In these chimeras, the donor and host differ by the minor H antigens associated with the C3H and Black strains, share the H-2^b MHC and the host bears additionally the H-2^k MHC. These chimeras were found to be specifically tolerant to the minor C3H antigens of the donor in the MHC context of donor H-2^b determinants. However the chimera was reactive to the donor minor H antigen in the context of the non-donor MHC (i.e., they were reactive to C3H [H-2^k]). Hence the tolerance pattern observed in this animal demonstrates that T cells are not tolerant to self minor H antigens alone but are only tolerant to these antigens in the MHC context in which they appear in the animal. 2) Macrophages are requisite for minor H specific CTL generation in vitro. 3) The minor antigens can be acquired, processed, and presented by macrophages. 4) The generation of minor H specific CTL by APC is inhibitable by the MT4 antibody.

Significance to Biomedical Research and the Program of the Institute: Insights into the mechanism of self-tolerance are important to our understanding of the function of the immune system. As the underlying principles become clarified, it is anticipated that they will have significant impact on human transplantation and the immunological approach to cancer treatment.

Proposed Course of the Project: To further elucidate the rate of antigen processing in the generation of CTL reactivity and tolerance to minor H antigens.

Publications: None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05108-03 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chereacters or less. Title must fit on one line between the borders.)*

T Cell Regulation of B Cell Activation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Hodes	Chief, Immunotherapy Section	IB, NCI
Others:	B. Needleman	Medical Staff Fellow	IB, NCI
	A. Finnegan	Investigator	IB, NCI
	K. Hathcock	Chemist	IB, NCI

COOPERATING UNITS *(if any)*

Department of Immunology, University of Tokyo, Tokyo, Japan
Department of Microbiology, Uniformed Services University of Health Sciences, Bethesda, MD

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It was previously demonstrated that B cell responses mediated by antigen specific T helper cells were regulated by T suppressor cells through two distinct MHC restricted pathways. It was subsequently demonstrated that cloned lines of Lyt 1^+ 2^- $L3T4^+$ antigen specific and MHC restricted suppressor cells could also mediate suppressor effector function in these T dependent antibody responses. Cloned T suppressor cells functioned to suppress the responses mediated by cloned T helper cells through an antigen specific mechanism requiring the tripartite, antigen-bridged interaction of T suppressor cells, T helper cells, and responding B cells.

Autoreactive T cell clones, specific for syngeneic I-A or I-E products were shown to function as T helper cells through two distinct pathways: One pathway was polyclonal and MHC unrestricted at the level of T helper-B cell interaction and the other was MHC restricted and dependent upon antigen specific triggering of responding B cells. It has been shown that MHC restricted, antigen-nonspecific suppressor populations which function to regulate responses by carrier specific T helper cells also function to regulate the responses mediated by autoreactive T helper cells. Activation of B cells by antigen specific and autoreactive T helper cells therefore appears to share susceptibility to similar regulatory influences.

The role of T cells in regulating the fine specificity of B cell antibody responses was studied by examining the T15 idiotype dominant response to phosphocholine (PC). It was found that cloned populations of carrier specific and MHC restricted T helper cells were capable of supporting T15 idiotype dominant responses in unprimed B cells of appropriate haplotype. These findings demonstrated that no absolute requirement exists for the participation of idiotype specific T_{H2} cells in the generation of optimally idiotype dominant responses in this experimental system.

Project Description

Objectives: This project is designed to evaluate the mechanisms of regulatory T cells controlling B cell activation and antibody response.

Methods Employed: The response systems studied are T cell and accessory cell dependent antibody response in vitro. Heterogeneous suppressor populations are generated by in vivo carrier priming of mice, followed by isolation of T cells by B cell and accessory cell depletion employing respectively treatment with a cytotoxic anti T cell reagent and passage over G-10 Sephadex columns. In vitro activation of suppressor cell activity is accomplished by culturing of T cells with accessory cells (T cell depleted irradiated spleen cells) and appropriate antigen. Following such activation, putative suppressor populations are assayed for their ability to affect the response of carrier primed T cells and hapten primed B cells to appropriate carrier-hapten soluble conjugates in vitro. Evaluation of T cell subpopulation requirements was accomplished by negative selection employing treatment with monoclonal anti-Lyt 1 or anti-Lyt 2 reagents plus complement. Assays of antigen-specific plaque forming cells were carried out.

The generation of monoclonal T cell populations was accomplished through repeated in vitro stimulation of in vivo primed T cells in the presence of antigen presenting cells, antigen, and a source of T cell growth factor. Cloning was accomplished through limiting dilution techniques, and cloned cells propogated through repeated in vitro stimulation. Antibody and idiotype responses to PC were studied by in vitro responses of heterogeneous or cloned T helper cells and unprimed B cells. Responses were assayed either by enzyme-linked immunosorbant assays (ELISA) or by the measurement of plaque forming cells (PFC).

Major Findings:

1. Suppressor T Cells.

The activation of T suppressor (T_S) cells requires in vivo priming, followed by in vitro re-exposure of T cells to the specific priming antigen in the presence of appropriate antigen presenting cells. Two apparently distinct suppressor pathways are activated by this procedure. Lyt 1^+2^- T cells are activated in vitro to generate Lyt 1^+2^- T_S cells which are capable of suppressing responses in an antigen non-specific manner, and require for their activity the presence of an unprimed Lyt 1^-2^+ population. In a second pathway, Lyt 1^-2^+ T cells from carrier primed animals are activated in vitro by specific carrier to generate Lyt 1^-2^+ T suppressor cells which then function in an antigen-specific effector pathway which does not appear to require the participation of additional T cell subpopulations.

The activation of both suppressor pathways identified above requires an MHC-restricted interaction between T_S precursors and accessory cells. The unique finding of these studies was that the suppressor populations are capable of suppressing only those antibody responses in which the functioning T helper cells expressed both the same antigen specificity and the same MHC restriction specificity as the suppressor cells regulating these responses. Cloned helper

and suppressor T cells have been generated, and cloned suppressor cells have been shown to function in an antigen specific and MHC restricted fashion, suppressing responses mediated both by heterogeneous T cell populations and by monoclonal T helper populations. These cells express a $Thy1^+$ $Lyt1^+$ $Lyt2^-$ $L3T4^+$ phenotype and proliferate in response to specific antigen in the context of appropriate I-A or I-E encoded restricting elements. The suppressor effector function of cloned suppressor cells was antigen specific. Suppression occurred only when the determinants recognized by the cloned T suppressor cell, the determinant recognized by the cloned T helper cell, and the hapten recognized by the responding B cell were all covalently linked to one another. It thus appeared that a tripartite antigen mediated interaction of these three cell populations was essential for suppression of B cell responses.

2. Autoreactive T Cells.

A series of T cell clones was generated by limiting dilution which were specific in their proliferative responses for syngeneic I-A or I-E products. Some of these autoreactive T cell clones functioned as T_H cells for B cell responses. The same cloned T_H cells could function through two different pathways. In one pathway, a predominantly IgM response was induced in unprimed B cells; this response was polyclonal and was MHC unrestricted at the level of T_H cell-B cell interaction. In the second pathway, an IgG response was induced in primed B cells only in the presence of the specific priming antigen and via an MHC-restricted T_H-B cell interaction. Heterogeneous populations of Lyt 1^+2^- T suppressor cells, generated as outlined above, were also found to suppress the B cell responses mediated by autoreactive T helper cells. This suppression was mediated in an MHC restricted fashion similar to that observed in the regulation of responses by carry specific T helper cells.

3. Augmenting T (T_A) cells.

Titrations of cloned T_H cells defined conditions for the generation of optimal B cell responses supported by these T_H cells. It was found that B cell responses could be enhanced or augmented above these levels by the addition of unprimed Lyt 1^+2^- T cells. These T_A cells were MHC-restricted in their function and augmented responses only when their MHC restriction specificity matched that of the cloned T_H cells functioning in a given response.

4. Idiotype regulation.

Both heterogeneous and cloned KLH specific T helper populations were capable of eliciting PC specific antibody responses from unprimed B cells in response to the antigen PC-KLH. The responses generated by cloned and heterogeneous T helper cells were comparable in magnitude, isotype (predominantly IgM) and affinity. In addition, the proportion of T15 idiotype positive antibodies generated were comparably high in response to either heterogeneous or cloned T helper cells. Similar findings were observed when antibody responses were assayed by either ELISA or PFC assays. Cloned carrier specific and MHC restricted T cells were therefore sufficient to generate optimally T15 idiotype dominant responses from unprimed B cells.

Significance to Biomedical Research and the Program of the Institute: The immune response to both experimental antigens and to naturally occuring antigens of infectious agents or of tumors is determined in large part by the regulatory influences of helper and suppressor T cell populations. It has been possible through the studies outlined above to analyze the regulatory effects on antibody responses in vitro. This has in turn permitted the analysis of T cell subpopulation interactions, and most recently the identification of monoclonal regulatory cells. It is anticipatated that through the further study of such populations, cellular as well as molecular analysis of immune response regulation can be accomplished. It is further intended that such principles will be applied to models of immune response to tumor antigens. The demonstrated function of self-reactive T cells raises clear questions about the relevance of such cells in vivo. The regulation of these cells in normal and pathologic autoimmune states is now a question of biologic importance.

Proposed Course of Project: Studies are now in progress analyzing the mechanism of suppression mediated by cloned T suppressor populations. Regulation of the responses of autoreactive T helper cells is also being examined. The regulation of idiotype dominance is being extended in studies of other antigen specific responses.

Publications:

Finnegan, A., Needleman, B., and Hodes, R. J.: 1984. Activation of B cells by autoreactive T cells. Cloned autoreactive T cells activate B cells by two distinct pathways. J. Immunol. 133:78-85.

Asano, Y. and Hodes, R. J.: 1984. T cell regulation of B cell activation. An antigen-mediated tripartite interaction of Ts cells, Th cells, and B cells is required for suppression. J. Immunol. 133:2864-2867.

Hodes, R. J.: 1985. Cloned Lyt-1^+, 2^- T suppressor cells. J. Molec. and Cell. Immunol. 2:14-16.

Hathcock, K. S., Kenny, J. J. and Hodes, R. J.: 1985. Helper T cell requirements for T15 idiotype expression of phosphocholine-specific antibodies. Eur. J. Immunol, in press.

Finnegan, A., Needleman, B. W., and Hodes R. J.: 1985. The function of autoreactive T cells in B cell activation. Survey of Immunologic Research, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05110-03 I

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immune Studies in Homosexual Men at Risk for Acquired Immune Deficiency Syndrome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. M. Shearer	Senior Investigator	IB, NCI
	H. B. Dickler	Senior Investigator	IB, NCI
Others:	W. Biddison	Senior Investigator	NINCDS
	S. Jacobson	Postdoctoral Fellow	NINCDS
	C. Via	Medical Staff Fellow	IB, NCI
	R. C. Gallo	Chief	LTCB, NCI

COOPERATING UNITS *(if any)*
K. S. Tung, Department of Pathology, University of New Mexico, Albuquerque, NM

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Peripheral blood leukocytes (PBL) were drawn from a number of age matched heterosexual and homosexual men from the Washington, DC area, and New Mexico. The PBL were sensitized in vitro to influenza virus and to HLA alloantigens. These sensitized cultures were tested for the generation of cytotoxic T lymphocytes (CTL) specific for influenza virus and alloantigens. Assays were also run for OKT4:OKT8 ratios (i.e., helper:suppressor cell), and for serum antibodies to HTLV-III as well as for the presence of HTLV-III virus. Similar studies were performed on AIDS patients and on donors with AIDS-related complex (ARC). Approximately 60% of homosexual men from both cities exhibited elevated CTL activity to HLA alloantigens. Among the AIDS and ARC patients, all exhibited a lack of CTL activity to influenza, although ~ 50% of these donors exhibited elevated CTL activity to alloantigens.

In vitro studies demonstrated that leukocytes from uninfected donors could be more efficiently infected with HTLV-III when the cultures were stimulated with HLA alloantigens than with T cell mitogens. This was particularly true in donors who exhibited elevated allogeneic CTL activity.

GPO 914-918

Project Description

Objectives: The purpose of this project is to investigate various immune parameters of individuals at high risk for AIDS. The immune parameters of individuals will be followed for at least two years to determine whether any of them develop AIDS symptoms. It is also the objective of this project to attempt to identify immunological abnormalities that may be predictive for AIDS development.

Methods Employed: Peripheral blood leukocytes were separated on Ficoll-Hypaque and sensitized in vitro to influenza self and HLA alloantigens. Proliferative and cytotoxic T lymphocyte (CTL) assays were run under optimal conditions. Suboptimal sensitization conditions were also tested by sensitizing with lower concentrations of influenza virus. OKT3, OKT4 and OKT8 analyses were run on the PBL using flow microfluorometry. Sera were tested for interferon and for antibodies that react with HLA typing cells. PBL from donors were also tested for retrovirus activity by reverse transcriptase activity, and sera were tested for antibodies to HTLV-III. Cultures of PBL were infected with HTLV-III after stimulation with PHA or alloantigens.

Major Findings: Approximately 70% of homosexual men appear to exhibit elevated T cell immunity to HLA alloantigens as measured by CTL testing, whereas approximately only 15% of heterosexual men exhibit elevated allogeneic CTL activity. Whether donors exhibited elevated CTL activity was not dependent on whether the donors were seropositive for HTLV-III. All donors with AIDS or with ARC exhibited lack of CTL activity to influenza recognized in association with self HLA antigens (self + X response), but many of the same donors exhibited elevated CTL to alloantigens. We were able, in some cases, to increase CTL to self + X by costimulation with influenza plus alloantigens. This raises the possibility of enhancing immunity to self + X in vivo in those AIDS patients who retain elevated T cell activity against alloantigens and provides a testable therapeutic approach to enhancing immunity in AIDS patients.

Since a high proportion of individuals in at least one group at risk for AIDS exhibit elevated CTL allogeneic activity, we tested whether HLA alloantigenic stimulation would augment in vitro infection of lymphocytes with HTLV-III, and whether PBL from "allo-primed" donors would be more efficiently infected with HTLV-III than PBL from "unprimed" donors. We found that stimulation with alloantigens was more efficient than stimulation with PHA (the usual stimulus used) for infection with HTLV-III. Furthermore, the allo-stimulated, virus-infected cultures grew longer and produced more virus than infected cultures stimulated with PHA. Finally, PBL from donors that were "allo-primed" were more easily infected in vitro after allostimulation then were PBL from unprimed donors.

Significance to Biomedical Research and the Program of the Institute: Understanding the etiology of AIDS is a high priority of the biomedical research community and particularly of the NCI. The evaluation of immune parameters of high risk groups may be predictive for AIDS development and may elucidate the mechanism(s) leading AIDS, and permit identification of susceptible individuals.

Proposed Course of Project: These donors will be followed for a period of up to two years with immune evaluation every three-to-six months. Individuals whose immune status appears to be of interest will be followed more extensively. Healthy homosexual donors who have antibodies to HTLV-III but no evidence for infection will be followed intensively for evidence for HTLV-III virus and changes in immune function. We shall attempt to "correct" and further study defects to self + X responses by co-stimulation with HLA alloantigens. We plan to develop a CTL response to HTLV-III antigens. We shall more extensively investigate the phenomenon of enhanced infection of PBL cultures after stimulation with HLA alloantigens, and attempt to determine whether elevated allogeneic T cell immune activity is a cofactor for AIDS susceptibility.

Publications:

Tung, K. S., Koster, F., Bernstein, D. C., Kriebel, P., Payne, S. M., and Shearer, G. M.: Elevated allogeneic cytotoxic T lymphocyte activity in peripheral blood leukocytes of homosexual men. J. Immunol., in press.

Shearer, G. M.: AIDS: An autoimmune pathologic model for the destruction of a subset of helper T lymphocytes. The Mount Sinai Journal of Medicine, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05111-03 I

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*
Generation of Allospecific CTL

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. McCarthy	Staff Fellow	IB, NCI
Others:	A. Singer	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

We have undertaken an immunogenetic analysis of the signals required for the primary in vitro generation of MHC class II-specific murine CTL. By varying the genetic relationship between responding and stimulating cells in mixed leukocyte cultures, we have demonstrated that optimal generation of class II-specific CTL requires the absence of a simultaneous class I MHC stimulus in the cultures. The inhibitory effect of a class I-specific response on the generation of class II-specific CTL in these studies: (i) is not due to preferential consumption of helper T cell-derived lymphokines by class I-specific cells; (ii) is not due to elimination of class II antigen-bearing stimulator cells by class I-specific cells; (iii) is not mediated via signals requiring linked recognition of class I and class II alloantigens on stimulator cells.

The inhibitory cell has been identified by depletion experiments as an $Lyt2^+$ cell in the responder spleen population. The action of this $Lyt2^+$ inhibitory cell can be blocked during culture by monoclonal α Lyt2 antibody in the absence of complement, which does not block the generation of class I-specific CTL. These results suggest that the class I-specific $Lyt2^+$ inhibitory cell detected in this system either is not a CTL, or is an Lyt2-independent CTL.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: The primary objective of this project is to identify the cellular interactions regulating the generation of cytolytic T lymphocytes specific for class II MHC alloantigens.

Methods Employed: Primary in vitro CTL responses were induced in mixed leukocyte cultures of spleen cells from H-2 congenic mouse strains. CTL activity generated during a five-day culture was assayed by lysis of ^{51}Cr-labeled LPS-activated target cells, which express high levels of class II MHC surface antigens. Regulatory cells in this system were characterized by negative selection with monoclonal-antibody-plus-complement treatment, prior to culture, or by functional blockade with monoclonal antibody in the five-day culture.

Major Findings: The development of reliable protocols for the generation of primary class II-specific CTL responses in vitro has allowed us to investigate the regulation of these responses. The simultaneous presence of an MHC class I stimulus precludes the development of optimal class II-specific CTL responses in mixed leukocyte culture. The "preclusion" phenomenon occurs in the presence (or absence) of exogeneous lymphokine-containing supernatants, which render the cultures independent of helper T cell activity. Competition for limiting amounts of lymphokines is therefore not a feasible mechanism by which preclusion might be affected. Preclusion of class II-specific CTL responses occurs in "three cell" cultures containing separate class I- and class II-disparate stimulator cell populations, as well as in cultures containing a single stimulator population presenting both genetic desparities to the responding cells. Since class I-specific responder cells in three cell cultures should not recognize the class II-disparate stimulators, competition for antigen or elimination of class II antigen-bearing cells by class I-specific responses is inadequate to explain the preclusion phenomenon. The three cell protocol results also indicate that the suppressive signals generated in these cultures apparently do not require linked recognition of stimulator-borne alloantigens by the class I-specific inhibitory cells and their class II-specific target cells.

We have used antibody-plus-complement depletion to demonstrate that preclusion of class II CTL responses is dependent upon Lyt2$^+$ cells in the responder population. Addition of small numbers of Lyt2$^+$ cells to Lyt2-depleted responder populations re-establishes preclusion in these cultures. The activity of Lyt 2$^+$ inhibitory cells can be blocked by inclusion of α Lyt2 monoclonal antibody in cultures containing both class I and class II stimuli. The Lyt2$^+$ class I-specific inhibitory cells are therefore Lyt2-dependent in their induction and/or their effector function. Class I-specific, Lyt2$^+$ CTL, in contrast, contain both Lyt2-dependent and Lyt2-independent subsets. The relationship between Lyt2-dependent inhibiting cells and CTL remains to be established.

Significance to Biomedical Research and the Program of the Institute: Identification of the regulatory elements responsible for immunodominance in the generation of allospecific CTL is necessary in the development of strategies for increasing CTL activity toward malignancies and decreasing or averting CTL responses toward allogeneic tissue grafts.

Proposed Course of Project: We anticipate extending these studies to protocols used in the in vivo generation of CTL and allograft rejection responses.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05112-03 I

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Analysis of Recognition Structures on T Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. A. Bluestone	Laboratory Leader	IB, NCI
Others:	D. H. Sachs	Chief, Transplantation Biology Section	IB, NCI
	O. Leo	Visiting Fellow	IB, NCI
	N. Shinohara	Expert	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION
Transplantation Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The recognition structures of T cells have been examined using anti-receptor antibodies and anti-T cell surface antigen specific antibodies prepared against cytotoxic T cell clones. Antisera produced in mice and rats against the CTL clones show specific reactivity and immunoprecipitation of receptor molecules on the cytotoxic T cells as well as other molecules which appear to be critical to T cell recognition. Present studies are designed to look at monoclonal antibodies prepared against the T cell receptor and other cell surface antigens. Three such monoclonal antibodies have been developed which specifically inhibit T cell function, precipitate glycoproteins of varying molecular weights, and are present on activated cytotoxic T cells but not precursor resting T cell populations. The antibodies generated are IgM, k. One antibody, 120-3-7-2, may recognize molecule similar to the T200 molecule while antibodies 120-4-24 and 128-12-17 recognize unique structures on the T cell surface which have not previously been described. In addition, a series of other monoclonal antibodies which recognize an Lyt6-linked molecule on the surface of the cytotoxic T cell clones has been generated. These monoclonal antibodies appear to subdivide class I specific CTL into two distinct populations. Current studies are underway to develop monoclonal antibodies against constant region portions of the T cell receptor as well as variable region determinants. In addition, further studies will be designed to analyze the monoclonal antibodies which have been developed and to better define the structural determinants involved in T cell function which are expressed on cytotoxic T cells.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: T cell responses to alloantigens encoded by the major histocompatibility complex are extremely important in immune responses associated with organ transplantation. Antibodies against structures expressed on alloreactive T cells could be useful in examining the immune response of T cells against alloantigens and might be employed both in vivo and in vitro to modify transplantation reactions and understand the repertoire of alloantigen specific T cells.

Methods Employed: Anti-T cell antibodies have been generated against several CTL clones specific for class I, K^b, MHC antigens. Mouse and rat anti-T cell antibodies were produced by immunizing repeatedly with 10^7 cytotoxic T cells on a weekly basis. Immune sera were examined on the immunizing clone for effects on CTL function as well as cell surface staining using the Fluorescence Activated Cell Sorter (FACSII). Immune sera were also examined for cross reactivity against monoclonal anti-H-2 antibodies directed at the same MHC class I antigens as that recognized by the CTL clones. The assay used is a binding site specific assay which examines the ability of test sera or monoclonal antibodies to inhibit the binding of anti-H-2 antibodies to H-2 bearing lymphocytes. The cloned CTL used in these studies were isolated from alloreactive mixed lymphocyte cultures. CTL were cloned by limiting dilution at 1 cell/well in the presence of stimulator cells and 10% T cell growth factor (IL-2), screened for cytolytic activity in a 4 hr ^{51}Cr-release assay and subcloned at 0.3 cells/well where appropriate. Fine specificity of the cloned CTL was examined using a panel of MHC congeneic mouse strains, MHC mutant mice, and immunoselected MHC mutant cell lines. Inhibition assays were performed by preincubating effector cells with the test reagent for 30 min prior to the ^{51}Cr release assay.

Major Findings: Alloreactive CTL clones have been used to immunize mice to generate antibodies against a T cell receptor molecule and other molecules associated with T cell function. In some instances, absorption with other CTL clones resulted in a clonotype specific antiserum. Antibodies have been identified in this serum which specifically inhibit the function of a series of CTL clones of different specificities and clearly react with determinants that are generally found on a large population of CTL. Three monoclonal antibodies have been derived from immunized mice, namely 120-4-24, 120-3-7-2, and 128-12-17. These three mouse monoclonal antibodies have been shown to specifically inhibit CTL function on a variety of CTL clones of different specificities. Further analysis of these monoclonal antibodies showed that the determinants detected are not expressed on resting spleen cells but are expressed after in vitro long term stimulation with allo class I but not class II antigens. Therefore, the expression of these molecules is clearly linked to the class specificity of this CTL. Biochemical analysis of the antigens detected by the monoclonal antibodies demonstrated that unique proteins are precipitated by each of these monoclonal antibodies. In the case of one monoclonal antibody, 120-3-7-2, the immunoprecipitated protein appears to be similar to a T200 molecule which has been previously described. However, the other two monoclonal antibodies appear to precipitate proteins that have not yet been described and must apparently be important in T cell recognition. Another class of monoclonal antibodies have also been derived which recognize

a molecule which is genetically linked to the Ly6 locus and likely to react with the Ly6 molecule. This molecule is expressed on resting T cells and on a subpopulation of thymus cells and on all class I specific CTL clones that have been analyzed. $L3T4^+$ class II specific proliferating and CTL clones do not express these markers. However, the $Ly2^+$ class II specific CTL clones do express Ly6. These monoclonal antibodies have been examined on bulk CTL populations and shown to be expressed on a subpopulation of alloreactive class I specific CTL. Current studies are underway to define the relationship between Ly6 expression and the repertoire of the class I specific CTL.

Significance to Biomedical Research and the Program of the Institute: A central problem in Transplantation Biology is the identification of specific methods to alter immune responses to transplantation antigens and thus effect allograft rejection. Since T cells are thought to be responsible for these immune reactions, reagents must be employed which affect the relevant T cell populations. One approach to this problem has been the use of anti-idiotypic antibodies against monoclonal anti-MHC antibodies to modify transplantation responses to alloantigens. These antibodies did not have a profound effect on T cell reactivity. Therefore new reagents against the relevant T cell populations are being developed which might be useful in modifying T cell responses to alloantigens.

Proposed Course of Research: Future studies will be designed to examine the precise nature of the antigenic determinants recognized by alloreactive cloned CTL. In addition, newly derived monoclonal anti-T cell antibodies will be examined to determine if any of these antibodies detect T cell receptor related molecules and might be useful in modifying transplantation responses. In addition, the newly derived monoclonal anti-T cell antibodies will be used to determine which T cells are indeed critical in responses to transplantation antigens in vivo.

Publications:

Sachs, D. H., Bluestone, J. A. and Epstein, S. L.: Anti-idiotype responses in transplantation immunity. Transplant. Proceed. 17:549-552, 1985.

Sachs, D. H., Bluestone, J. A., and Epstein, S. L.: Idiotypes of anti-MHC receptors. In Streilein, J. W., Ahmad, F., Black, S., Blomberg, B. and Voellmy, R. W. (Eds.): Advances in Gene Technology: Molecular Biology of the Immune System, Cambridge, Cambridge University Press, 1985, pp. 101-104.

Bluestone, J. A., Sunshine, J. L. and Sachs, D. H.: Idiotypes on anti-MHC antibodies: Detection of a public idiotype on anti-H-$2K^b$ antibodies. In Yamamure, Y. and Tada, T. (Eds.): Progress in Immunology V. Academic Press, Japan, Inc., 1984, pp. 1505-1515.

Bluestone, J. A., Auchincloss, H., Jr., Epstein, S. L. and Sachs, D. H.: Idiotypes of anti-MHC monoclonal antibodies. In Kohler, H., Urbain, J., and Cazenave, P.-A. (Eds.): Idiotype, in biology and Medicine. Academic Press, Inc., 1984, pp. 243-269.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05114-02 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Sequence Organization of Class I Major Histocompatibility Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. S. Singer	Senior Investigator	IB, NCI
Others:	S. Rudikoff	Senior Investigator	LG, NCI
	M. L. Satz	Visiting Fellow	IB, NCI
	R. Ehrlich	Visiting Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The aim of this work is to determine the DNA sequence organization of class I genes contained in the swine major histocompatibility complex (SLA). Both genomic lambda and cosmid libraries have been constructed from a homozygous SLA^{dd} animal. From these, two non-allelic porcine class I SLA genes have been isolated and characterized. Both genes are expressed in mouse L cells, directing the synthesis of class I SLA molecules which carry common monomorphic determinants, but are serologically distinct. The corresponding DNA sequences have been determined. The organization of both of these genes is similar to that of other class I genes; consisting of 8 exons encoding a leader polypeptide, three extra cellular domains, a transmembrane and intracytoplasmic domain. The two genes are highly homologous in both exon and intron segments, with average homologies of 88 and 80%, respectively. Nucleotide changes in exon 2 are clustered, whereas those in the other exons are dispersed throughout. Comparison of the swine DNA sequences with class I genes from other species reveals a generally high conservation of exons 2, 3, 4 and 6. The data indicate an order of swine>human>rabbit>mouse in the relationship of class I genes.

Project Description

Objectives: This laboratory has undertaken a study of the molecular biology of the class I genes of the major histocompatibility locus of the miniature swine. It is known that the antigens encoded by these genes are highly polymorphic and responsible for both graft rejection and regulation of immune responses. Biochemical studies have demonstrated that the MHC antigens of mouse, man, and miniature swine are structurally homologous. As a result of studies of the MHC genes of mouse and man, others have proposed various mechanisms for the generation of polymorphism in this multigene family. The aim of this study is to attempt to assess the various selective pressures exerted on MHC genes during their evolution, including those operating at the allelic and species levels. In addition, we hope to gain further insights into the generation of polymorphism in this multigene family, since the number of class I MHC genes in the miniature swine is far more limited than in either man or mouse.

Methods Employed: Recombinant genomic DNA libraries were constructed in the lambda phage vector, Charon 4A, and the cosmid vector, pHC 79, using purified 20-40 kb pig DNA fragments, generated from partial restriction enzyme digests of pig liver DNA. Genomic clones containing class I MHC genes were identified by hybridization of radiolabelled probes, either human or swine, with the libraries. Once genomic clones are characterized as containing MHC genes, a detailed analysis of the DNA sequence organization of the clone is conducted. Segments of the isolated DNA are subcloned into plasmid vectors; subclones containing coding DNA sequences are then subjected to restriction enzyme mapping and direct DNA sequence analysis. Two methods of DNA sequence analysis are employed: the chemical degradation method described by Maxam and Gilbert and M13 subcloning coupled with dideoxy, enzymatic sequencing. Data are analyzed by a variety of available computer programs. The limits of the transcriptional unit have been mapped by appropriate exonuclease VII-protection experiments.

Major Findings: The two characterized class I SLA genes are both expressed in mouse L cells, indicating that they are functional, and direct the synthesis of SLA molecules which carry common monomorphic determinants, but which are serologically distinct. The two genes are highly homologous in both the exon (88%) and intron (80%) segments. The gene organization consists of 8 exons encoding a leader domain, three extracellular domains, a transmembrane domain, cytoplasmic domain and 3' untranslated region. This organization is similar to those reported in other species. Putative regulatory sequences can be identified in the 5' flanking regions of both genes. A 'CAT' box (CCAAT) is found 5' of the initiation codon. Approximately 25 bp. down-stream from the 'CAT' box, one gene contains a canonical 'TATA' box (TATAA). In the same position, the other gene contains the sequence TCTAA, which is also found in the human HLA A3 gene. Since exonuclease VII mapping studies indicate that transcription begins in this region, these data suggest that the presence of either sequence, TCTAA or TATAA, can result in proper transcription.

Of the protein encoding exons, only the exon encoding the leader, exon 1, shows size variation. Exon 2, which encodes the first extracellular domain of the native molecule is the least homologous of the 3 exons encoding extracellular domains. Alterations in exon 2 are clustered, consistent with the possibility that they have arisen by gene conversion mechanisms. Homologies between the

two genes are also high (96-100%) in exons 5-8.

Comparison of the swine DNA sequences with class I genes from other species reveals a generally high conservation of exons 2, 3, 4, and 6 with lower homology in the remaining protein encoding domains. Introls are significantly less well conserved, although moderate homology is found between swine and human class I MHC genes in both introls and 3' flanking regions. Taken together, with comparisons of the deduced protein sequences, these data indicate an order of swine>human>rabbit>mouse in the relationship of class I genes.

Significance to Biomedical Research and the Program of the Institute: Genetic and cellular studies in a number of mammals, including man, mouse, and pig have demonstrated that class I MHC antigens play an integral role in the cellular interactions leading to both humoral and cellular immunity. In particular, the ability to respond to viral infection and to accept tissue grafts appears to be determined by the class I MHC antigens. Despite the clear importance of the major histocompatibility locus in the immune response, relatively little is known at the molecular level about the content or genetic organization of this multigene family. An understanding of the molecular variations of MHC genes and their corresponding antigens may afford the possibility of treating various immunodeficiency diseases by appropriate genetic manipulations.

Proposed Course of Project: Characterization of the structure of the swine MHC genes will proceed along the following lines. From the cosmid and phage libraries, it has been possible to identify the remaining members of the class I SVA family. A distantly related member of the family has been chosen for further DNA sequence analysis in an effort to gain insights into the mechanisms which generate diversity. In addition, we plan to focus on potential regulatory loci at the 5' ends of the various class I genes. Such studies should reveal common sequences necessary for the regulation of expression of multigene family, as well as identify unique regions required for the differential expression of these genes.

Publications:

Satz, M. S., Wang, L-C., Singer, D. S., Rudikoff, S.: 1985. Structure and expression of two porcine genomic clones encoding class I MHC antigens. J. Immunol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05115-02 I

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Expression of Class I MHC Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Neme, title, leboratory, and institute affiliation)*

PI:	D. S. Singer	Senior Investigator	IB, NCI
Others:	R. Ehrlich	Visiting Fellow	IB, NCI
	H. Golding	Visiting Fellow	IB, NCI
	L. Abelson	Biologist	IB, NCI
	L. Satz	Visiting Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.8	3.8	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors B

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The aim of this work is to investigate the mechanisms controlling the expression of a multigene family, namely the class I MHC genes. It has been demonstrated that there are 6-8 class I genes in the genome of the miniature swine. The products of some of these genes are classical transplantation antigens, whereas the others are unknown. Although the transplantation antigens appear to be expressed on all somatic cells, the level of this expression varies. The patterns of expression of the remaining genes have not been analyzed. To address the question of the molecular regulation of the expression of this set of related genes, a series of genomic clones containing MHC-homologous DNA sequences has been isolated. Of the 6-8 genes in the genome, six have been isolated. The expression potential of these genes has been analyzed by transfection of mouse L cells. Two categories of MHC genes have been identified in this way: 1) a set of closely related genes which are expressed in L cells and appear to represent the genes encoding the classical transplantation antigens. These genes encode products which are expressed on the cell surface and are able to bind a monoclonal antibody which recognizes a common determinant. 2) A set of more distantly related genes which are also expressed in L cells, but whose product is not recognized by the monoclonal antibody. Regulatory sequences within one of the SLA genes have been identified by generating a series of 5' end deletion mutants. Using such a set of mutants, the positions of the transcriptional promoter and interferon - enhancer have been mapped. Other segments of the class I gene are also being analyzed for the presence of other regulatory sequences. The patterns of expression of the various class I genes in vivo has also been analyzed. Large variations in the level of expression are observed among different tissues for all of the genes. Representative genes from each of the two class I gene sub-groups appear to have similar patterns of expression.

Project Description

Objectives: This laboratory has undertaken to study the molecular biology of class I genes in the major histocompatibility locus in the miniature swine. These genes constitute a multigene family of only 6-8 members in the genome of the pig. It is known from serological studies in the mouse that class I antigens fall into two categories: transplantation antigens, which are constitutively expressed on nearly all somatic cell types, albeit to different extents, and differentiation antigens, such as T1, expressed primarily on one tissue type. Thus, although the members of this family are closely related with respect to DNA sequence, and clearly evolved from a common primordial ancestor, they are subject to different regulatory constraints. Therefore, this multigene family provides an excellent system in which to study the regulation of expression of its members. Two concurrent approaches to this problem are being used: 1) The expression of individual pig MHC genes is being studied in mouse L cells transfected with various genomic MHC clones. An analysis of the regulatory constraints operating in this model system will provide insights into the regulation of expression of a single member of the multigene family. Comparisons of MHC genes which are or are not expressed in these cells will further identify important regulatory regions. 2) The patterns of expression of MHC genes in pig tissues will also be assessed. These studies will allow us to classify genes as either transplantation antigen encoding or differentiation antigen encoding. It will then be possible to examine both the DNA sequences and regulatory factors responsible for differential expression.

Methods Employed: Individual MHC clones have been isolated from either phage or cosmid genomic libraries by established techniques. Segments of pig DNA containing MHC-homologous sequences are isolated and purified; mouse Ltk^- cells are cotransformed with these DNA preparations and HSV tk genes. Selected transfectants containing pig DNA sequences are then assessed for expression of SLA antigens on the mouse cell surface and for SLA RNA expression. Expression of SLA antigens is monitored by indirect immunofluorescence, using a monoclonal anti-SLA antibody. RNA expression is monitored by hybridization of purified total RNA from transfectant cell lines with appropriate probes. To test for the presence of regulatory sequences, swine MHC genes have been truncated at their 5' or 3' ends and reassayed in mouse L cells for SLA expression. More refined mapping of regulatory sequences is accomplished by generating deletion mutants using Bal 31 exonuclease. Mutants thus generated are then ligated to test expression vectors to monitor regulatory and promotor activity by transfection into mouse L cells.

In vivo expression of class I genes is assessed by analyzing total RNA prepared from a variety of tissues. Analysis is either by direct Northern blot hybridization or by S1 nuclease.

Major Findings: Studies on the regulation of expression of class I MHC genes have focused primarily on one genomic clone, PD1. It has been demonstrated that mouse L cells transfected with PD1 express SLA antigen on their cell surfaces. This expression results from the selective transcription of SLA coding sequences; contiguous pig DNA sequences are not transcribed. To

further characterize the DNA sequences which regulate this expression, a variety of deletion mutants have been analyzed. Trucation of 5' flanking sequences of the SLA gene results in the loss of transcription of SLA sequences. Bal 31 mapping of the 5' flanking sequences has located the SLA promoter region, as follows. A series of Bal 31 deletion mutants were generated spanning a region of 1 kb. at the 5' end of the SLA gene. Each of these mutants was attached to the bacterial chloramphonicol acetly transferase (CAT) gene and its ability to promote CAT transcription assessed.

Treatment of SLA-transfectants with mouse interferon results in a marked increase in the levels of surface SLA^d and in the rate of gene transcription. Using the same set of Bal 31 deletion mutants described above, it has been possible to map the ultimate target of the interferon effect to the 5' flanking region of the SLA gene.

Analysis of RNA from a variety of tissues indicates that expression of at least three of the SLA genes is regulated and apparently in similar fashion. In all three cases, different levels of RNA are observed in different tissues, ranging from very little expression in testis and thymus to high levels of expression in lymphoid tissues. Thus, SLA gene expression appears to be regulated. The molecular basis of this expression is currently under further investigation.

Significance to Biomedical Research and the Program of the Institute: Studies in a number of species, including the miniature swine, have demonstrated the importance of products of MHC genes in normal immune responses. Products of class I MHC genes control cellular responses leading to graft rejection and cellular immunity to virus. Products of other class I MHC genes are differentiation antigens. The expression of these different antigens can be modulated by a variety of exogenous factors, such as interferon or viral proteins, both of which may alter the immune response. Despite the importance of the MHC in the immune response, nothing is known at the molecular level about the regulation of expression of this multigene family. An understanding of the molecular basis of MHC gene expression may afford the possibility of developing therapeutic approaches which could appropriately modulate MHC gene expression to counteract the deleterious effect of infectious agents.

Proposed Course of Project: Studies on the regulation of MHC gene expression will proceed along the following lines:

(1) The regulation of class I MHC gene expression in mouse L cells will be further studied. The Bal 31 mutants will be assessed for the presence of enhancer sequences. Further characterization of the promoter region will be approached by promoter shuffling experiments between genes with different patterns of expression.

(2) Studies on the differential patterns of class I MHC gene expression in tissues will also be pursued. A variety of lymphoid and non-lymphoid pig cell lines are available, as well as pig interferon. It will therefore be possible to assess the expression of the SLA genes in homologous cells. This should allow the identification of regulatory sequences. Once such DNA segments have been identified, it should be possible to isolate associated factors and characterize their tissue specificity and interactions with DNA.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05116-02 I

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Graft-Versus-Host Disease Prophylaxis in Allogenic Bone Marrow Transplantation

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. E. Gress	Senior Investigator	IB, NCI
Others:	R. R. Quinones	Medical Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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☒(a) Human subjects ☒(b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Efforts are being directed towards the prevention or control of graft-versus-host disease in human allogeneic bone marrow transplantation. Since such graft-versus-host disease is mediated by alloreactive T cells in the inoculated marrow, reagents and techniques have been developed to remove these T cells from the marrow inoculum and to measure the success of that depletion. To this end, several murine monoclonal antibodies specific for antigens expressed on human T cells have been developed, three of which are cytotoxic. These antibodies have been untilized, in conjunction with other depletion techniques, for complement-mediated lysis of T cells in marrow. By a clonogenic assay now available, residual T cells in marrow following such a depletion are at a level of less than 0.01% of the total cell population. With respect to the control of alloreactive T cells mediating graft-versus-host disease, studies on the origin or generation of such alloreactivity have been undertaken in murine radiation bone marrow chimeras. It has been shown that the generation is influenced by a unique interaction of T cell genotype and the T cell maturation environment. The fine specificity of human CTL has been demonstrated to be sufficient to distinguish among alpha 1 and alpha 2 domain changes of class I major histocompatibility complex molecules. Such mature alloreactive human cytotoxic T cells have been further studied with respect to cell surface molecules utilized in their interactions with target cells as the basis for therapeutic interventions with the intent of preventing tissue damage mediated by such alloreactive cytotoxic T cells.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: 1). To produce monoclonal antibodies directed against human T cell specific antigens; 2). To develop techniques utilizing these antibodies to deplete human T cells from marrow; 3). To apply these approaches for the prevention of graft-versus-host disease in human allogeneic bone marrow transplantation across major HLA barriers; 4). To establish a bank of characterized, T cell depleted marrow for use in human bone marrow transplantation; 5). To study the origin of the basis for graft-versus-host disease, the generation of the T cell allorepertoire; 6). To determine methods for the control of T cell mediated alloresponses by the use of monoclonal antibodies directed at cell surface molecules.

Methods Employed: Mice are immunized with human thymocytes and peripheral (mature) T cells. Hybridoma cell lines are obtained by fusion of immune mouse splenic lymphocytes with mouse myeloma cells. The cells are fused by exposure to polyethelene glycol and then cultured in selective HAT medium for two weeks. Hybridoma cells secreting anti-T cell antibodies are detected by assays for binding to plates coated with human T cells (cell-bound ELISA) and by complement-mediated cytotoxicity. Positive hybridoma cell lines are cloned. The monoclonal antibodies so produced are further characterized by differential binding and cytotoxicity on purified lymphocyte subpopulations. Such analysis includes two color immunoflourescence analysis by the Fluorescence Activated Cell Sorter utilizing biotinylated antibodies with avidin conjugates and directly fluoresceinated antibodies. Large amounts of monoclonal antibodies are raised in culture supernatants or by passage in vivo in mice to produce ascites hybridoma antibodies.

To further define the specificity of the monoclonal antibodies, the cell surface molecules for which they are specific are isolated and characterized. Cell surface proteins are labeled with ^{125}I. Labeled cells are solubilized by detergent lysis and large particle are removed by ultracentrifugation. Radiolabeled determinants are reacted with monoclonal antibodies and precipitated by Staphloccocal aureus. Molecular weight is determined by polyacrylamide gel electrophoresis.

Hybridoma antibodies specific for T cell antigens are purified following determination of the antibody subclass by radial immunodiffusion (Ochterlony reaction) with nylon wool, precipitation by ammonium sulfate (dialysis) passage over DEAE, gel separation and sizing. These characterized, purified antibodies are then utilized in T cell depletions. Initial titering for depletion by complement-mediated lysis is carried out on ^{51}Cr-labeled T cell targets with varying concentrations of antibody and complement. Antibody and complement at optimal titer are then utilized alone or in combination with other depletion techniques to determine relative efficacy of various depletion regimens. The effect of the T cell content in the starting, untreated cell population on the final residual T cell number after depletion treatment is assessed by applying the depletion procedure to cell populations with varying T cell content (peripheral blood lymphocytes, aspirated marrow and surgically resected marrow). Marrows with low numbers of contaminating T cells are harvested by surical resection, prepared as cell suspensions in RPMI medium, T cell depleted by the optimal combination of depletion procedures utilizing

antibody, complement and erythrocyte rosetting and cryopreserved for human transplantation. Completeness of depletion is assessed by proliferation assays (mitogen and mixed lymphocyte culture responses) and by clonogenic assay.

To study T cell allorepertoire generation, murine bone marrow chimeras are prepared by lethal irradiation of recipient mice and reconstituted with T cell depleted marrow from allogeneic donors. Cytolytic T cell activity is assayed in vitro by four hour ^{51}Cr release.

To further study regulation of T cell alloresponses and xenogeneic responses, human T cell clones with specificity for alloantigens or xenoantigens are generated by stimulation with allogeneic or xenogeneic cells and IL2 under conditions of limiting dilution and then expanded to the numbers needed. Specificity is assessed by cytotoxic cell activity against transfected L cells, in vivo generated mutants, or in vitro generated mutants. Cell surface molecule interactions are investigated by the use of monoclonal antibodies with specificity for LFA-1, LFA-2, T3 or T8.

Major Findings: Fusion of murine myeloma cells with mouse spleen cells immunized against human T cells has resulted in eight clones, six with specificity for human T cells. Of these, three are stable hybridomas producing cytotoxic antibodies. Two of these antibodies are IgG2 and one is IgM. One antibody is blocked in its binding by the monoclonal 9.6 and inhibits E-rosette formation; by these criteria it is therefore specific for the T cell E-rosette receptor. It is pan-thymocyte in distribution, inhibits killing of allogeneic targets by cytotoxic T cells, inhibits T cell proliferation induced by allogeneic determinants and is negative on Rhesus monkey lymphocytes. Another is pan-T in its distribution. It reacts with a subset of thymocytes, does not inhibit cytotoxic T cells, and is positive on Rhesus monkey lymphocytes. Preliminary evidence from immunoprecipitation studies indicates that this antibody is specific for the T cell antigen receptor.

Optimal T cell depletion of heavily T cell contaminated populations results when antibody plus complement lysis is preceeded by E-rosetting. Two cycles of complement are distinctly superior to one. Surgically resected marrow with one to five percent contamination by cells bearing mature T cell markers has fewer residual T cells after a fixed depletion regimen of E-rosetting followed by antibody plus complement than aspirated (conventionally harvested) marrow with ten to thirty percent contamination by mature T cells. Resected marrow after T cell depletion has a residual T cell content of less than 0.01% by the clonogenic assay. This assay detects as few as 1 to 2 T cells per $1x10^6$ marrow cells. Marrow can be surgically resected from organ donors with yields of greater than 10^{10} cells which is adequate for consideration of clinical application.

The $\underline{H\text{-}2}^b$ T cell repertoire specific for the allodeterminants encoded by the $\underline{K}^b$ mutant bm6 results from a unique interaction of T cell genotype and T cell maturation environment. This interaction involves gene products encoded by the $\underline{K}$ region with the result that $\underline{K}$ region homology is required for the generation of the repertoire. This raises the possibility of intramolecular associative recognition requirements in T cell responses to alloantigens. Alloreactive

T cells specific for class I determinants can be cloned directly from an unsen-unsensitized peripheral human lymphocyte population and expanded to numbers sufficient to study mechanisms of alloreactivity and recognition of allogeneic target cells. Human T cells have sufficient specificity to distinguish alpha 1 vs. alpha 2 domain associated antigens of class I major histocompatibility complex molecules. Their interactions with target cells is marked by a variable rather than fixed role for LFA 1, LFA 2, T3 and the "C" epitope of T8. Preliminary results indicate that monoclonal antibodies directed at LFA 1 block human CTL associated tissue damage in vivo.

Significance to Biomedical Research and the Program of the Institute: Monoclonal antibodies directed against human T cell specific antigens can be used to probe the biological functions of these antigens and to eliminate T cells bearing these antigens from marrow for the purpose of preventing graft-versus-host disease in human allogeneic bone marrow transplantation.

The prevention of graft-versus-host disease and the establishment of a bank of characterized T cell depleted marrow for use in clinical bone marrow transplantation will provide a significant, new potential for the use of allogeneic bone marrow transplantations as a treatment modality for a variety of clinical diseases including neoplasms, inborn errors of metabolism and hemoglobinopathies. An understanding of the generation of the T cell allo repertoire is intertwined with issues of T cell recognition of alloantigens. Control of such alloresponses which are the basis of graft-versus-host disease will depend on an understanding of T cell function and recognition requirements. The mechanisms of target cell/alloreactive T cell interactions provides a model of the formulation of new therapeutic approaches aimed at preventing the tissue damage associated with the interaction of human CTL with their targets.

Proposed Course of Project: Efforts will continue to generate monoclonal antibodies which are T cell specific in their activity. The biological relevance of the T cell antigens towards which such antibodies are directed will be assessed in a variety of systems such as T cell proliferative responses and T cell mediated cytotoxicity.

Techniques for depletion of T cells from marrow will be further expanded and refined. The use of plant toxins conjugated to monoclonal antibodies is being investigated. Further collaborations will be carried out to expand the studies of allogeneic bone marrow transplantation in animal models in mouse, pig and rhesus monkey. A bank of T cell depleted marrows is being established with characterization as to HLA typing, extent of T cell depletion and viability of bone marrow progenitor cells.

Banked marrows will be utilized in clinical application in the treatment of patients with neoplasms. This will involve patients in the pediatric age group. Efforts will continue in the use of murine radiation bone marrow chimeras for studies on the generation of the T cell allorepertoire and T cell recognition of alloantigens. Alloreactive T cell clones will be utilized for the study of the cellular interactions necessary for tissue injury.

Publications:

Ryan, J. J., R. E. Gress, K. S. Hathcock, and R. J. Hodes: Recognition and response to alloantigens in vivo. II. Priming with accessory cell depleted allogenic splenocytes: Generation of specific unresponsiveness to foreign major histocompatibility complex determinants. J. Immunol. 133:2343, 1984.

Lynch, D. H., R. E. Gress, B. W. Needleman, S. A. Rosenberg, and R. J. Hodes: T cell responses to Mls determinants are restricted by cross reactive MHC determinants. J. Immunol. 134:2071, 1985.

Sharpe, T. A., R. E. Gress, D. H. Sachs, and S. A. Rosenberg: Assessment of mixed lymphocyte reactivity and human bone marrow cell cultures. Transplantation 1985, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05117-02 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Allodeterminants of Class I Major Histocompatibility Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: J. A. Bluestone Laboratory Leader IB, NCI

COOPERATING UNITS *(if any)*

S. G. Nathenson and S. Geier, Dept. Microbiology & Immunology, Albert Einstein Col. of Med., Bronx, NY; H. Allen and R. A. Flavell, Biogen Research Corp., Cambridge, MA; David Margulies, Laboratory of Immunology, NAIAD

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Current efforts have been devoted to examining the nature of the allo-determinants recognized by cloned T cell populations as compared to those determinants recognized by alloantibodies. To examine this question, H-2 structural mutants have been isolated from a somatic cell line by mutagenesis and immunoselection using monoclonal anti-H-2 antibodies. Examination of alloantigen-specific CTL clones on these mutants suggest that the majority of CTL clones recognize determinants different from those which elicit antibody production. Analysis of the in vitro-derived mutants has shown that new determinants are created by the mAb immunoselection procedure which are recognized by cytotoxic T cells. In addition, the regions of the MHC molecule involved in CTL recognition were studied using L cells transfected with H-2 genes constructed by shuffling exons between the H-2K^b and D^b genes. Other altered MHC class I genes have also been examined including L cells which have been transfected with truncated L^d and D^d genes and express only the α3/TM portion of the molecule and L cells transfected with hybrid MHC genes between mouse and human class I genes. The findings suggest that unlike mAbs which can recognize individual epitopes on different domains, CTL recognition is influenced by the interaction of the two external domains, do not recognize determinants in the α3 domain on the intact MHC molecule but require α3 (human or mouse) for CTL recognition.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: The generation of monoclonal alloreactive T cell populations to: (1) Identify the regions of class I MHC heavy chains that are responsible for the alloantigenicity of these molecules; (2) investigate the activation requirements of T cells; and (3) determine the role of conformation of the H-2 molecule in induction and blocking of alloresponses.

Methods Employed: Cloned CTL were isolated from alloreactive and modified-self-specific mixed lymphocyte cultures. CTL were cloned by limiting dilution at 1 cell/well in the presence of stimulator cells and 10% T cell growth factor (IL-2) and screened for cytolytic activity in a 4 hour ^{51}Cr release assay. Fine specificity of the cloned CTL was examined using a panel of MHC congenic mouse strains and in some cases MHC mutant mice.

Somatic cell lines from which mutants were selected were derived from F_1 neonatal bone marrow cells transformed with Abelson virus. Monoclonal antibodies reacting with different epitope clusters were used to immunoselect H-2 expressing tumor cells. Mutagenized cells were treated repeatedly with a mAb plus complement. The minority of residual cells which expressed normal amounts of cell surface MHC were positively selected by fluorescence activated cell sorting using a pool of mAbs and subcloned using soft agar. Clones with reduced reactivity with the selecting antibody but near normal reactivity with other anti-H-2 mAbs were used.

The hybrid class I genes were constructed by reciprocal exon exchanges between the K^b and D^b genes. The resultant constructs which had their α_1 domains exchanged were transfected into L cells. These transformed cell lines expressed a polypeptide with domain α_1 of K^b and all other domains from D^b (K^b1/D^b) or reciprocally domain α_1 of D^b and all other domains from K^b (D^b1/K^b). Additional recombinant H-2 class I genes were constructed by deletion of the $\alpha3$ domain of the K^b molecule. These transformed cell lines expressed a smaller polypeptide with the $\alpha1$ and $\alpha2$ domains apparently unaltered. Transfected recombinant HLA-A2 and H-$2K^b$ genes were also expressed in mouse L cells and a human rhabdosarcoma cell. Finally, H-$2L^d$ and H-$2D^d$ genes were altered by deleting the $\alpha1$ and $\alpha2$ domains. The truncated gene was transfected into L cells and shown to be expressed on the L cell surface.

Cytolytic T cell reactivity was examined in a ^{51}Cr-release assay. Target cells were radiolabeled with ^{51} Chromate solution. Effector cells were added and the mixture incubated for 4 hrs. The supernatants were collected and percentage lysis was calculated. Skin graft analysis was performed by transplanting full thickness tail skin to recipient mice. The grafts were assessed for rejection on a daily basis.

Major Findings: In vitro-derived H-$2K^b$ structural variant lines were studied using cytotoxic T cell (CTL) clones generated from different alloreactive combinations and monoclonal antibodies (mAbs) which reacted with different H-$2K^b$ domains. The H-$2K^b$ structural mutants were derived from a mutagenized somatic cell line (R8) using an α_1 or an α_2 domain specific mAb as the immunoselecting reagents. The determinants recognized by the CTL clones

generated in different allogeneic combinations were altered in some of these mutant cell lines. It is clear from the analysis that the determinants recognized by the CTL and mAbs are different. However, there appears to be some conformational changes which affect both specificities. mAb blocking studies suggested that inhibition of CTL activity by anti-H-2K^b mAbs does not necessarily reflect the fine specificity of ther CTL activity. Finally, neo-determinants were identified on the in vitro-derived somatic varients. CTLs generated from a wild type C57BL/10 anti-H-2K^{bm3}, H-2K^{bm23} or H-2K^{bm6} mutant CML reaction lysed two of the R8 mutants, R8.24 and R8.246 but did not lyse the wild type R8 cells. Additional evidence suggests that one amino acid, No. 77, has been altered in the R8.24 mutant. This amino acid has also been altered in the H-2^{bm3} and H-2^{bm23} in vivo mutant mice and may suggest a highly relevant site for CTL recognition.

To identify the regions of class I antigens that are involved in CTL recognition, >15 mAbs and >20 CTL clones specific for either H-2K^b or H-2D^b have been examined on the H-2 hybrid antigens (K^b1/D^b and D^b1/K^b) expressed on transfected L cells. The pattern of CTL and mAb recognition suggests that the interaction of the first two domains influences the allogeneic determinants. Although the binding of all but one of the mAbs could be mapped to one of the external domains, α_1 or α_2, the binding affinity in several instances was markedly reduced. By comparison, none of the CTL clones or bulk populations examined lysed the transfected cells, suggesting that either the affinity of the CTL recognition was reduced sufficiently to obviate lysis or the determinants recognized by the CTL were sufficiently altered to prevent CTL recognition. In addition, these experiments suggested that the majority of class I specific CTL were directed against the α1 and α2 domains of the H-2K^b and D^b molecules. This was confirmed by the fact that a majority of H-2K^b specific CTL clones recognized H-2K^b/HLA-A2 recombinant MHC molecules, in which the α1 andα2 domains were H-2K^b and the α3 domain was HLA-A2. Surprisingly, a greater influence of CTL recognition was found using the human fibroblast line vs the mouse L cell line for transfecting the hybrid molecules. Several clones which recognized K^b/A2 in L cells did not recognize this molecule in the human rhabdosarcoma line. Deletion of the α3 domain also abrogated CTL recognition. Additional studies using truncated H-2D^d and H-2L^d transfected L cells showed that specific CTL could be generated against α3 domain of the class I molecule.

The MHC regions involved in the rejection of skin grafts was examined. Mice were primed, in vivo, with L cells transfected with hybrid MHC molecules (L^d/D^d). Skin graft rejection was assessed across a class I-only L^d difference. The findings suggested that the α1 and α2 domains were most important in skin graft rejection and the immune cells which mediate the rejection recognize conformational determinants dependent on both the α1 and α2 domains.

Significance to Biomedical Research and the Program of the Institute:
Alloreactive T cell responses provide the major barrier to successful organ transplantation. In order to specifically modulate alloreactivity a greater understanding of the nature of allorecognition is required. These studies suggest that allodeterminants recognized by cytolytic T cells differ from those recognized by alloantibodies. In addition, recognition depends heavily on the conformation of the class I molecule.

Proposed Course of Research: New, H-2 mutants and class I hybrid gene constructs will be examined to further define the polymorphic structures on MHC molecules involved in allorecognition.

Publications:

Bluestone, J. A., Potter, T. A., Chatterjee-Hasrouni, S., and Rajan, T. V.: CTL recognize different determinants from those defined serologically on L^d somatic cell mutants. J. Immunol. 133:1168-1173, 1984.

Levy, R. B., Ozato, K., Richman, J. C. and Bluestone, J. A.: Molecular localization of allogeneic and self determinants recognized by bulk and clonal populaitons of cytotoxic T-cells. J. Immunol. 134:1342-1348, 1985

Bluestone, J. A., Palman, C., Foo, M., Geier, S. G. and Nathenson, S.: Analysis of major histocompatibility complex class I antigens using in vitro-derived somatic cell mutants. In Sercarz, E., Cantor, H. and Chess, L. (Eds.): Regulation of the Immune System, UCLA Ortho Meeting, 1984, Vol. 18:89.

Bluestone, J. A., Foo, M., Allen, H. and Flavell, R. A.: Allospecific cytolytic T lymphocytes recognize conformational determinants on hybrid mouse transplantation antigens. J. Exp. Med., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05118-02 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Immune Response to Tumor Cells and Alloantigen

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. C. Ting	Senior Investigator	IB, NCI
Others:	M. E. Hargrove	Microbiologist	IB, NCI
	T. R. Malek	Investigator	LI, NIAID
	D. Winkler	Biologist	IB, NCI
	J. Wunderlich	Senior Investigator	IB, NCI
	S. S. Yang	Senior Investigator	LCO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. Regulation and control of immune responses: Two differentiation factors were generated in syngeneic lymphocyte-macrophage cultures in which there was no requirement of antigenic or mitogenic stimulation for their production. These two factors were CCDF (cytotoxic cell differentiation factor) and TCDF (T cell differentiation factor). CCDF was required to induce the differentiation of IL-2 activated precursors into LICC (lymphokine induced cytotoxic cells). TCDF was required to induce the differentiation of antigen-or mitogen-activated CTL precursors into CTL effectors. In addition, TCDF was needed to maintain the cytotoxic activity of the fully generated CTL.

2. Tumor Immunology: LICC generated by lymphokines (IL-2 and CCDF) selectively killed lymphoid and solid tumor targets of different H-2 haplotypes and of different etiological origins. The LICC were also found to be very effective in preventing the in vivo growth of both lymphoid and solid tumors. The ability to generate LICC was preserved in the tumor bearing hosts until the terminal stage of tumor growth. The inability to generate LICC at this late state was not caused by deficient lymphokine production; rather the generation of suppressor T cells interferred with LICC induction. LICC appears to be one of the tumor bearing host's uncompromised defense mechanisms against tumor.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: 1) Regulation of immune responses to tumor cells; 2) Regulation of immune responses to alloantigen; 3) Regulation of lymphokine production for the generation of cytotoxic lymphocytes.

Methods Employed: 1) Tumor cells used in experiments: Tumor cells transformed by oncogenic viruses, chemicals or unknown agents are kept in tissue culture or maintained as transplanted tumors in mice; 2) In vitro cell-mediated immunity was determined by the ^{125}IUdR release assay; 3) In vivo tumor immunity was determined by the tumor neutralization experiments; 4) In vitro generation of cytotoxic effectors. The allogeneic mixed lymphocyte cultures were used to generate the alloreactive cytotoxic T lymphocytes (CTL), the lymphokine-induced cytotoxic cells (LICC) were generated either by culturing normal spleen cells with syngeneic peritoneal cells and indomethacin, or by culturing normal spleen cells with interleukin 2 (IL2) and cytotoxic cell differentiation factors (CCDF); 5) Lymphokine production: Purified Interleukin 1 (IL1) was purchased from Genzyme (Boston, MA). CCDF was prepared by culturing mouse peritoneal cells with indomethacin. TCDF (T cell differentiation factor) was prepared by culturing normal mouse spleen cells with peritoneal macrophages and indomethacin. Purified human recombinant IL2 was obtained from Dr. Steven A. Rosenberg (Surgery Branch, NCI).

Major Findings:

1. Regulation and Control of Immune Responses:

A. Lymphokine regulation of cell-mediated cytotoxic responses to tumor cells: Lymphokine-induced cytotoxic cells (LICC) were generated directly by culturing normal spleen cells with lymphokines, or were generated indirectly by culturing normal spleen cells with syngeneic peritoneal macrophages and indomethacin. At least two lymphokines were involved: (1) interleukin 2 (IL2) which was produced by T cells, and (2) cytotoxic cell differentiation factor (CCDF) which was produced primarily by macrophages. IL2 was needed to activate the LICC precursors which were shown to have a Thy1$^+$, Lyt2$^-$ and Asialo GM1$^+$ phenotype. CCDF was needed to induce the differentiation of IL2-activated precursors into LICC effectors which were shown to have a Thy1$^+$, Lyt2$^-$ and Asialo GM1$^-$ phenotype. LICC selectively killed tumor targets of different H-2 haplotypes and of different etiological origins.

B. Lymphokine regulation of allogeneic MHC antigen-induced or mitogen-induced CTL responses: In the absence of antigenic or mitogenic stimulation, a T cell differentiation factor (TCDF) was produced in cultures containing normal spleen cells, syngeneic macrophages and indomethacin. This TCDF is required for the differentiaiton of alloantigen or mitogen-activated CTL precursors into CTL effectors. It was further shown that for the generation of CTL, both IL2 and TCDF were required. Once CTL were fully generated, supplementation of TCDF alone was sufficient to maintain the cytotoxic activity of CTL, and there was no requirement for supplementation with exogenous IL2.

C. Regulation of lymphokine production by MHC molecules: Both CCDF and TCDF were produced in the syngeneic lymphocyte-macrophage cultures. There was no requirement for antigenic or mitogenic stimulation. CCDF was required for the generation of LICC which selectively killed tumor targets; whereas TCDF was required for the generation of antigen or mitogen-induced CTL. Recognition of self Ia molecules was essential for the initiation of TCDF production. Addition of monoclonal anti IA antibody to lymphokine-producing cultures specifically blocked the TCDF production in appropriate H-2 haplotypes of cells. Self KD molecules did not seem to be involved in these reactions, insofar as TCDF production was not affected by monoclonal anti KD antibody.

2. Tumor Immunology: The LICC generated by lymphokines (IL2 and CCDF) selectively killed lymphoid and solid tumor targets in vitro. To determine the in vivo anti tumor activity of these LICC, they were generated in B6 ($H\text{-}2^b$) spleen cells and then tested by the tumor neutralization experiments (Winn assay) in vivo. Two syngeneic tumors were used: a Friend virus-induced leukemia FBL-3 and a methylcholanthrene-induced sarcoma MC1. The LICC were found to be very effective in preventing the growth of both lymphoid and solid tumors. The cells responsible for conferring in vivo protection were $Thy1^+$. To further determine whether LICC might be involved in the host defense against tumor growth, the ability of the tumor bearing hosts to generate LICC was tested. It was found that the ability to generate LICC was preserved until the terminal stage of tumor growth. The inability to generate LICC at this stage was not caused by deficient lymphokine production, because peritoneal macrophages obtained from tumor bearing hosts were able to induce LICC generation in normal spleen cells. At the terminal stage of tumor growth, suppressor T cells of $Lyt2^+$ phenotype were present and these suppressor T cells interferred with LICC induction. These results suggest that LICC is one of the tumor bearing host's uncompromised defense mechanisms against tumor.

Significance to Biomedical Research and the Program of the Institute:

1. The lymphokine-induced cytotoxic cells (LICC) not only were very effective in mediating in vitro killing of various lymphoid or solid tumor targets, they were also very effective in preventing the in vivo growth of both lymphoid and solid tumors. Furthermore, the LICC precursors were present in tumor bearing hosts and in vitro readily generated LICC effectors. Since LICC were generated in the absence of antigenic stimulation, they presumably could be induced in hosts with non immunogenic tumors, such as most spontaneous neoplasms. Therefore, arming the pathway to generate LICC may prove to be very effective in retarding the progression of tumor growth or tumor metastasis.

2. TCDF and CCDF were produced in the absence of antigenic or mitogenic stimulation. Recognition of self Ia molecules appeared to be an early step in initiating the production of these lymphokines. This may prove to be a new pathway for lymphokine production. To examine the mechanism for the production of these growth factors or lymphokines should help to understand the different pathways for the generation of CTL or LICC. It may also help to understand the activation process for oncogenesis.

Proposed Course of Research:

1. Regulation and control of immune responses: lymphokine regulation of the generation of CTL and LICC.

2. Mechanism for the production of TCDF in syngeneic systems.

3. Generation of LICC in tumor bearing hosts and its implication in cancer immunotherapy.

4. Purification of TCDF and CCDF.

Publications:

Yang, S. S., Malek, T. R., Hargrove, M. E., and Ting, C. C.: Lymphokine-induced cytotoxicity: Requirement of two lymphokines for the generation of optimal cytotoxic response. J. Immunol. in press (1985).

Ting, C. C., Wunderlich, J. R., Hargrove, M. E., and Winkler, D.: In vitro and in vivo anti tumor activity of lymphokine-induced cytotoxic cells. Int. J. Cancer. in press (1985).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05119-02 I

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Role of Helper T Cells in Allogeneic Responses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Mizuochi	Visiting Fellow	IB, NCI
Others:	A. Singer	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the spece provided.)*

Precursors of class I specific allo-CTL were found to be activated by at least two distinct populations of helper T cells: (1) $L3T4^+$ $Lyt2^-$ helper T cells which were class II restricted, and (2) $L3T4^-$ $Lyt2^+$ helper T cells which were class I restricted. The mechanism by which these two T_H populations functioned were distinct since monoclonal antibody against the IL-2 receptors expressed by pCTL preferentially blocked the activation of pCTL by class II restricted helper T cells rather than class I restricted helper T cell. Thus, these results demonstrate the existence of two different classes of T_H cells and two distinct helper mechanisms in the induction of class I specific allo-CTL responses.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives: The major objective of this project is to define the function, recognition specificity and cell surface phenotype of helper T cells involved in the induction of class I specific allo-CTL responses.

Methods Employed: In vitro generation of CTL: Two ml mixed lymphocyte cultures consisted of: a) $4x10^6$ responder spleen cells, b) $4x10^6$ stimulator cells that had been irradiated with 2000R, and c) $4x10^6$ T-depleted and 2000R irradiated spleen cells as a source of accessory cells. After 5 days of culture, cytotoxic activities of effector cells were assayed on the relevant ConA blast target cells by standard 4 hr ^{51}Cr release assay.

IL-2 assay: Culture supernatants were assayed for IL-2 by their ability to support the proliferation of the IL-2 dependent T cell line (HT-2). Proliferation was assessed by the incorporation of ^3H-thymidine.

Major Findings:

1. Allospecific CTL responses require the activation of either class I or class II restricted T_H cells. The class I restricted T_H cells are $L3T4^-$ $Lyt2^+$ whereas the class II restricted T_H cells are $L3T4^+$ $Lyt2^-$.

2. Monoclonal antibodies against the murine IL-2 receptor (7D4) expressed by pCTL block their activation by class II-restricted T_H cells but not by class I restricted T_H cells suggesting that the Ia-dependent pathway is highly dependent on exogenously produced IL-2 but that the Ia-independent pathway is not.

3. Monoclonal antibodies against the L3T4 determinant blocks the activation of class II restricted T_H cells whereas anti-Lyt2 antibody blocks the activation of class I restricted T_H cells.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the T-helper mechanisms which initiate alloreactions should provide us with the tools to selectively augment or decrease immune reactivity in various clinical states.

Proposed Course of the Project: In the future, this project will be directed at investigating how class I restricted helper T cells are triggered and how their specificity is determined.

Publications:

Mizuochi, T., Golding, H., Rosenberg, A. S., Glimcher, C. H., Malek, T. R., and Singer, A.: Both $L3T4^+$ and $Lyt2^+$ T-helper cells initiate CTL responses against allogeneic MHC antigens but not against TNP-self. J. Exp. Med., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05120-02 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Regulation of Lymphocyte Proliferation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K. Kelly	Senior Investigator	IB, NCI
Others:	M. Kearns	Junior Staff Fellow	IB, NCI
	S. Irving	Guest Researcher	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Lymphocyte metabolism and effector function expression are regulated by antigen/mitogen and lymphokine binding to cell surface receptors. We are investigating the physiological consequences of mitogen and lymphokine mediated signals by isolating and characterizing genes which are transcriptionally regulated by these events. We expect that genes induced within a few hours after antigen or mitogen activation of lymphocytes will be fundamentally important for the initiation of proliferation and effector function expression in these cells. We have shown that the c-myc oncogene is transcriptionally induced as early as one hour after the activation of murine B cells with LPS or T cells with Con A. Two additional proto-oncogenes, c-myb and c-fos, are similarly regulated by IL-2 and PHA binding, respectively, to human T lymphocytes. Thus, three oncogenes are members of the gene family regulated by mitogen binding to the surface of lymphocytes. The identification and characterization of additional members of this inducible gene family is currently in progress utilizing PHA stimulated human peripheral blood T cells, subtraction cDNA cloning methodology, and DNA-mediated gene transfer.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: The broad objectives of this laboratory concern the isolation and characterization of those gene sequences that are transcriptionally activated by mitogen binding to T lymphocytes. Characterization of inducible gene sequences will include 1) structural analysis and positive identification with known gene sequences/gene products where possible and 2) analysis of induced genes for regulatory properties such as kinetics of induction, cell cycle expression and the mechanism of RNA level increases. An additional objective of characterization is the functional analysis of induced gene sequences. Such functional analysis will be accomplished by DNA-mediated gene transfer of expression-competent cDNA clones into nontransformed lymphocytes maintained in vitro.

Methods Employed: We are utilizing recombinant DNA technology that is useful in the manipulation of eucaryotic RNA and DNA. Some of the specific methods employed include subtraction cDNA cloning, genomic cloning, Northern and Southern blot analyses, S1 nuclease analyses, and chromatin DNAase hypersensitivity. The methodology employed for DNA-mediated gene transfer includes DEAE-Dextran transfection, electroporation, and standard tissue culture techniques.

Major Findings: As a new laboratory, much of our effort in the past six months has concerned the development of new techniques and systems (sections B and C) that will form the necessary basis for future investigation.

A. The progression of T cells from GO to DNA synthesis requires at least two temporally separated growth signals; one initial signal resulting from antigen or mitogen binding, and the other later signal from interleukin 2 binding. The effects of such activation signals appear to be mediated in large part by the expression of novel gene sequences. The steady-state RNA levels of three proto-oncogenes, c-myc, c-fos, and c-myb, are elevated very soon after the delivery of growth signals to T cells. In human peripheral blood lymphocytes, c-myc and c-fos RNA levels are induced within 30 and 90 minutes, respectively, in response to PHA or OKT3 binding. On the other hand, c-myc and c-myb RNA transcripts increase within 60 minutes following IL-2 binding, while c-fos expression is not induced. The elevation of these gene transcripts occurs in the presence of cycloheximide, indicating a mechanism of regulation independent of protein synthesis. As shown by run-on transcription assays, the increased levels of c-myc and c-fos RNA result at least in part from increased transcriptional initiation. Immunoprecipitation of ^{35}S-methionine labeled extracts from PHA activated PBL show a concordant increase in mRNA and protein for c-myc and c-fos. The proteins encoded by c-myc, c-fos, and c-myb are localized in the nucleus.

B. The functional role of c-myc and c-myb induction following growth signal delivery to T lymphocytes is being assayed by DNA-mediated gene transfer. The murine c-myc and v-myb structural genes have been manipulated by recombinant DNA techniques to place the expression of these genes under the control of an exogenously regulatable promotor, the murine metalothinein promotor. Following the introduction of such cloned genes into cells, the level of c-myc or v-myb mRNA is controlled by the concentration of heavy

metals in the exogenous media. The above gene construcitons have been introduced by DEAE-Dextran mediated transient gene transfer into cloned murine cytotoxic T cells. A variety of parameters for this technique have been optimized for each T cell clone. Initial results indicate that oncogene transfected cells generally show increased proliferative capacity as assayed by ^{3}H-Thymidine incorporation 48 to 72 hours after gene transfer. In addition, 50 uM $ZnCl_2$ increased ^{3}H-Thymidine incorporation in oncogene transfected cells while control vector transfected cells remain unchanged. Currently, the levels of mRNA resulting from the transcription of exogenously introduced DNA are being determined. In this way, the relationship between increased proliferative capacity and c-myc mRNA levels in normal T cells can be assayed in a quantitative way.

C. A fundamental question concerning lymphocyte activation is the identification of those gene sequences that are transcriptionally regulated following mitogen or antigen signalling. We are currently isolating cDNA clones that are induced in T lymphocytes within two hours after PHA stimulation.

The ability to isolate an inducible gene depends upon the differential expression of that gene in induced and noninduced messenger RNA populations. Although it is expected that the gene induced as a result of mitogen stimulation of T cells will represent a very small percentage of the total messenger RNA population in induced cells, these gene sequences can be identified in a cDNA library with a labeled probe prepared by subtraction hybridization. A labeled cDNA probe representative of the entire mRNA population in induced cells can be repeatedly hybridized to mRNA from noninduced cells, resulting in the production of cDNA-mRNA hybrid molecules. Hybrids can be easily separated from the single stranded, labeled cDNA probe, allowing isolation and enrichment of the probe for specifically induced genes. Identical sequences in the cDNA library can then be selected out using the labeled probe.

Large quantities of mitogen-induced and noninduced RNA have been isolated from human PBL, obtained by leukophoresis. T cells have been isolated by ficoll hypaque gradient centrifugation and subsequent nylon wool purification. Following in vitro stimulation, RNA has been purified from such T cell populations and has been demonstrated to be representative of an activated population by the characteristic expression of inducible oncogene mRNA. The cloning procedure employed to produce cDNA copies of the activated mRNA population is the Okayama-Berg System (pCDV1/pL1) that features 1) an enzymatic selection system for full-length reverse transcripts and 2) a cloning vector that permit direct expression in mammalian cells.

cDNA cloning requires numerous enzymatic steps and the preparation of enzymatically modified purified vector starting materials. We currently have successfully prepared the vector starting materials and have demonstrated reverse transcription of the purified mRNA, homopolynucleotide tailing, and second strand synthesis. The completion of a cDNA library is anticipated within a period of weeks.

Significance to Biomedical Research and the Program of the Institute: Essential to an understanding of the immune response are the intracellular biochemical events which result from antigen and lymphokine binding. We are utilizing an approach based on recombinant DNA technology to define a critically important consequence of lymphocyte activation, the identity of transcriptionally-activated gene sequences. In addition, the physiological mechanisms which initiate and maintain proliferation in lymphocytes are fundamental to the field of growth regulation and cancer biology. Recent advances in this field include the identification of gene sequences, oncogenes, whose increased expression or unregulated expression override normal cellular growth control mechanisms, leading to malignant transformation. By understanding the normal biochemical events associated with proliferation in lymphocytes, one should gain insight into the regulation and cell cycle associated role of oncogenes. Finally, defining genes which are involved in the induction of differentiated functions is of central significance to an understanding of gene regulation in general.

Proposed Course of Project: cDNA library construction is in progress with anticipated completion within weeks. Thereafter, production of a subtracted, ^{32}P-labeled probe will proceed with anticipated completion within the month. Subsequent selection, isolation, and characterization of positive clones will proceed by standard procedures.

Publications:

Armelin, H. A., Armelin, M. C. S., Kelly, K., Stewart, T., Leder, P., Cochran, B. H. and Stiles, C. D.: A functional role for c-myc in the mitogenic response to platelet-derived growth factor. Nature 310:655, 1984.

Kelly, K., Cochran, B., Stiles, C. D., and Leder, P.: Cell cycle control of c-myc expression. In Proceedings on RNA Tumor Viruses in Human Cancer. M. Rich (Ed.), pp 67-75, 1984.

Ju, S. T., Siebenlist, U., Kelly, K.: Effects of V-lambda gene diversity on generation of antigen-specific lymphocytes. J. Immunol. 133:3378-3382, 1984.

Kelly, K. and Siebenlist, U.: The role of c-myc in the proliferation of normal and neoplastic cells. J. Clin. Immun. 5:65, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05121-01 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of the T Cell Repertoire to Class I-like Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Keene	Post-doctoral Fellow	IB, NCI
Others:	A. Singer	Senior Investigator	IB, NCI

COOPERATING UNITS *(If any)*

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Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

In order to better understand the mechanisms by which the T cell repertoire is generated, the T cell response to Qa region MHC antigens was analyzed with respect to the recognition capabilities of the various functional T cell subpopulations. The results of these studies indicate that Qa specific CTL possess a typical class I specific repertoire in that they recognize self plus X and do not require intentional in vivo priming if adequate help is provided. In contrast, unlike the helper T cell response to most class I antigens, Qa specific $Lyt2^+$ helper T cells could not be detected. It is concluded that although Qa-region antigens differ from other class I MHC antigens in that they have been reported to be 1) expressed on the cell surface in lower amounts, 2) are not extensively polymorphic, and 3) appear to be expressed predominantly on cells of the hematopoetic lineage, the Qa-specific CTL repertoire does not appear to be unique. The uniqueness of the Qa-specific helper cell repertoire remains to be determined.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: The objective of this project is to examine the T cell repertoire to Qa-region MHC antigens.

Methods Employed: Thymocytes and spleen cells from Qa-1 congenic mice were analyzed for the ability to proliferate, produce IL-2 and/or generate CTL to Qa-1 disparate stimulator cells. Anti-L3T4 and/or anti-Lyt2 monoclonal antibodies were used either as blocking antibodies or in the presence of complement to examine each of the various Qa-1 specific T cell subpopulations.

Major Findings: By the following criteria, the CTL which recognize Qa-region antigens were found to possess a repertoire similar to other class I specific CTL. 1) Anti-Qa-1 CTL were shown to recognize self plus X by their cross-reaction on self-TNP. 2) Similar to other class I specific CTL, anti-Qa-1 CTL were found not to require intentional in vivo priming in the presence of adequate help. 3) Anti-Qa-1 CTL were found in both the spleen and thymus, although indirect evidence suggests that the precursor frequency may be lower for anti-Qa-1 CTL than other class I specific CTL in these organs. In contrast, the helper T cell repertoire to Qa-region antigens was found to be unlike that of most class I MHC antigens in that no $Lyt2^+$ Qa-specific helper T cells were detected even in multiply primed mice.

Significance to Biomedical Research and the Program of the Institute: An understanding of how the T cell repertoire is generated will allow insights into the mechanisms responsible for the regulation of T lymphocyte responses to foreign antigens and thereby yield ways to manipulate immune disorders which are a consequence of regulatory failure.

Proposed Course of Project: The future aim of the project is to further characterize helper cell defect to Qa region MHC antigens. Particular emphasis will be placed on 1) examining the role of class II restricted $L3T4^+$ helper T cells in the generation of Qa specific CTL, 2) examining the defect in $Lyt2^+$ Qa specific helper T cells with regard to graft rejection, and 3) determining whether the ability of $Lyt2^+$ helper T cells to recognize Qa region antigens is under genetic control influenced by thymic phenotype.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05122-01 I

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chereters or less. Title must fit on one line between the borders.)*
Mechanisms of Allograft Rejection

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, end institute affiliation)*

PI:	A. Rosenberg	Medical Staff Fellow	IB, NCI
Others:	A. Singer	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NIC, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A subset of $Lyt2^+$ cells has been shown to possess helper activity. The activation of these cells is contingent upon the presence of an allo class I difference and serves to activate CTLs directed against the same class I antigen. There are no data that currently support the existence of $Lyt2^+$ T helpers responsive to Minor H antigens or TNP lated cells. We have recently noted a striking correlation between the frequency of $Lyt2^+$ T helpers specific for the class I differences defined by the Kb mutant series and the rate of skin graft rejection of these mutants by B6 mice. Specifically, rapid rejection of bml skin (different from B6 by 3 amino acid substitutions in the K^b moiety) by B6 animals is noted whereas bm6 skin (also different from B6 by several amino acid changes in the K^b region) is either rejected extremely slowly or fails to reject. When precursor frequencies of various subpopulations are examined, a striking discrepancy is noted in the frequency of the $Lyt2^+$ T helper with that directed against bml being 1/36,000 and that directed against bm6 being $1/>2x10^6$. The precursor frequencies of the $L3T4^+$ T helpers and that of the CTLs are of the same order of magnitude. We are therefore attempting to clarify the role of these cells in the rejection of various types of skin grafts, specifically class I, class II, and minor only different grafts. Preliminary results indicate that $Lyt2^+$ cells are responsible for rejection of class I only different grafts whereas $L3T4^+$ cells appear to be necessary for rejection of class II only different grafts.

Project Description

Objectives: To study the role of $Lyt2^+$ T helper cell subset in allograft rejection.

Methods Employed: We utilized an adoptive transfer model consisting of athymic (nude) mice skin grafted and then injected with variously treated cell populations. Specifically, skin grafts (either syngeneic, minor different, class I only different, or class II only different) were placed on day 1 and the following day mice were injected with $50x10^6$ spleen cells from normal B10animals that had been either Lyt2 + C' treated, L3T4 + C' treated or untreated. The bandages were removed after 7 days and the skin grafts read daily.

Major Findings: B10 nude mice engrafted with bm1 or bm12 (class I vs class II different) skin showed a markedly discrepant pattern of response when injected with varying cell populaitons. Those engrafted with bm1 skin rejected rapidly when injected with L3T4 + C' treated cells whereas those engrafted with bm12 did not reject when injected with these cells. However, when Lyt2 + C' treated cells were injected, the reverse pattern was seen with rapid rejection of the class II different skin and markedly delayed or no rejection of the class I only different skin. Hence, it would appear that as concerns rapid graft rejection, $Lyt2^+$ cells are responsible for rejection of class I different grafts and $L3T4^+$ cells are essential for rejection of class II different grafts.

Significance to Biomedical Research and the Program of the Institute: The $Lyt2^+$ T helper cell may well play a critical role in the rejection of mismatched allografts as well as a role in tumor rejection. Further understanding may lead to methods to disrupt its function in allograft rejection and enhance its response to tumors.

Proposed Course of Project: To further elucidate the role of both $Lyt2^+$ and $L3T4^+$ cells in the rejection of allografts.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05123-01 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Isolation and Characterization of a Novel Class I MHC Gene

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. S. Singer	Senior Investigator	IB, NCI
Others:	R. Ehrlich	Visiting Fellow	IB, NCI

COOPERATING UNITS *(if any)*

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SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In all species, the class I MHC genes consist of a family of homologous DNA sequences, some of which encode the heavy chain moiety of the classical transplantation antigens. The functions of the remaining class I genes and their products are poorly, or not at all, understood. In the miniature swine, which contains only 6-8 class I homologous DNA sequences, two closely related genes encoding classical transplantation antigens have been isolated and characterized. A third sequence, which is highly divergent, has now also been isolated and is being characterized. By molecular hybridization to genomic DNA and direct DNA sequence analysis, it has been demonstrated that this gene, PD6, is a member of the limited class I SLA family. Despite extensive divergence, it appears to share the over-all sequence organization of a class I gene. The PD6 gene is expressed both in transfected mouse L cells and in vivo.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives: This laboratory has undertaken to study the members of the class I MHC gene family in the miniature swine, with the aim of understanding their organization and function. Unlike other species such as mouse and man whose class I gene families number 20-40 members, the pig appears to have a restricted family of only 6-8 members. Of these, 3-5 appear to be closely related, whereas the remainder are more distantly related. The closely related members contain those genes encoding classical transplantation antigens. The purposes of the present studies were to determine: 1) whether the distantly related DNA sequences were indeed members of the class I gene family or of another family and 2) whether these divergent sequences could be expressed and if so the nature of their products. In the mouse, the antigens Qa and T1, thought to be differentiation antigens, are encoded by members of the class I gene family. Similar differentiation antigen-encoding genes have not yet been characterized in the pig and might be expected to be found among the more distantly related sequences. Such analyses will reveal the genomic organization of the class I family and the maximum number of class I genes necessary for a normal immune response, as well as the functional spectrum of class I-encoded products.

Methods Employed: The clone, PD6, which contains a divergent class I SLA DNA sequence, was isolated from a phage genomic library by cross-hybridization with another SLA gene. The class I-homologous DNA sequences have been characterized by standard techniques: restriction enzyme mapping, molecular hybridization with another SLA gene and sub-genic fragments, and direct DNA sequence analysis (using the M13/dodeoxy method of Sanger). Expression of the PD6 sequences has been assessed in both mouse L cells following DNA-mediated gene transfer and in vivo by direct filter hybridization of the gene to RNA derived from various tissues or by S1 nuclease analyses of the same RNA samples.

Major Findings: The recombinant clone, PD6, contains an SLA-homologous DNA segment which is highly divergent from other class I DNA sequences. However, it is a member of this multigene family, and not of another distantly related family. DNA sequence analysis and direct hybridization have indicated that PD6 has a sequence organization similar to other class I genes. Analysis of the 5' end of the gene revealed homology in both the 5' flanking region and first exon. A conserved region around the 'CAT' box was observed, but no 'TATA' box was found. Sequencing of part of exon 4 revealed marked divergence (65%) from other SLA genes, which are highly (96%) conserved in this region. Open reading frames were found in the exons analyzed, consistent with the possibility that PD6 is a functional gene, not a pseudogene.

PD6 is expressed both in transfected mouse L cells and in vivo, as assessed by the presence of PD6-specific RNA. In vivo, PD6 RNA is found in a variety of lymphoid and non-lymphoid tissues, with the highest levels in spleen, lymph node and liver.

Significance to Biomedical Research and the Program of the Institute: Studies in a number of mammals, including man, mouse and pig have demonstrated the importance of products of some class I MHC genes in normal immune responses,

including control of graft rejection and cellular immunity to virus. The role of the remaining members of the class I MHC gene family is not known. The present studies will help define the functions, if any, of these gene products and should establish the minimum genetic requirements for a normal immune response. In addition, comparison of the DNA sequences of the class I MHC genes will provide information about the evolution and generation of diversity of this multigene family.

Proposed Course of Project: Studies on the PD6 class I MHC gene will proceed along the following lines: 1) The nature of the product of the PD6 gene, if any, will be investigated. In vitro translation of PD6-specific mRNA will provide insights into the size of the product encoded, and whether PD6 can be expressed in protein. If so, attempts will be made to generate antisera specific for the PD6 product using either transfected mouse L cells or synthetic oligopeptides. Such antisera will afford the possibility of studying the cell surface distribution of the PD6 product as well as its role in immune function. 2) DNA sequence analysis of other exons of PD6 will continue in order to generate information on the evolution of this gene.

Publications:

Ehrlich, R., Lifshitz, R., Pescovitz, M., and Singer, D.: Isolation and characterization of a novel class I MHC gene. Submitted.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05124-01 I

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Expression and Function of a Porcine Class I MHC Gene in Transgenic Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. Singer	Senior Investigator	IB, NCI
Others:	J. Bluestone	Laboratory Leader	IB, NCI
	R. Hodes	Chief, Immunotherapy Section	IB, NCI
	M. Pescovitz	Senior Staff Fellow	IB, NCI
	W. Frels	Agricultural Research Service	USDA

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A porcine class I major histocompatibility complex (SLA) gene has been introduced into the genome of a C57BL/10 mouse. This transgenic mouse expressed SLA antigen on its cell surfaces and transmitted the gene to off-spring, in which the gene is also expressed. Skin grafts of such transgenic mice were rejected by normal C57BL/10 mice, suggesting that the foreign SLA antigen expressed in the transgenic mice is recognized as a functional transplantation antigen.

GPO 914-918

Project Description

Objectives: The introduction of isolated class I MHC genes into cultured mammalian cells has been used to study their expression, antigenicity and antigen-presenting capacity. This approach allows the introduction of genes not normally expressed in the recipient cells, such as foreign, mutant or hybrid MHC genes constructed in vitro. In addition, the expression of individual members of a multigene family can be studied in isolation. However, such studies have been generally limited to a small number of cell types, such as L cells, and the questions addressed have been limited to those related to the expression of the introduced gene in that particular cellular environment. It has not been possible to address developmental issues such as the patterns of tissue-specific expression of individual class I MHC gene or the influence of newly introduced genes or the induction of self tolerance and the maturation of the immune response. To study these unanswered questions, a cloned class I SLA gene was microinjected into the fertilized eggs of mice.

Methods Employed: Male pronuclei of one-cell C57BL/10 (B10) fertilized eggs were injected either with approximately 500 copies of a 9 kb Hind III fragment derived directly from a genomic SLA clone, PD1, or with about 2000 copies of a 5.6 Kb Hind III-Bam HI fragment, subcloned into pBR322 and linearized with either Hind III or Eco RI. These fragments of swine DNA each contain the entire SLA gene. To test for swine DNA sequences, DNA was extracted from a segment of tail of each of the mice and hybridized with an SLA DNA probe. Expression of SLA antigen on the cell surface was assessed by cell-surface labeling of peripheral blood lymphocytes (PBL) with a monoclonal antibody specific for SLA determinants, and analysis by flow microfluorometry.

Major Findings: Of 171 eggs injected with the 5.6 kb Hind III-Bam HI DNA fragment, 100 were transferred to pseudopregnant females, and there were 63 off-spring. None were positive for the SLA gene. Twenty-seven eggs were injected with the 9 kb Hind III DNA fragment and 20 were transferred to a single foster mother; there were 5 off-spring. A single SLA-positive mouse was identified. PBL from this animal were shown to express SLA antigen on their cell surfaces. In an effort to examine the stability of expression and heritability of the SLA gene and to estimate the number of loci into which the SLA gene integrated, this animal was mated to B10 females. All off-spring which contained the SLA gene expressed SLA antigen on their cell surfaces. It was estimated that there is only one, or a few copies, of the SLA gene in the genome of these mice. It is concluded that the SLA gene is in a single integration site and is transmitted to progeny in accordance with Mendelian inheritance. SLA expression in transgenic mice may be regulated: different levels of SLA antigen expression are observed in spleen, lymph node, bone marrow and thymus. The ability of the SLA antigen to be recognized as a transplantation antigen was assessed by engrafting normal B10 animals with tail skin from transgenic and normal B10 mice. All recipients rejected skin from transgenic mice, but not skin from normal mice.

Significance to Biomedical Research and the Program of the Institute: Genetic and cellular studies in a number of mammals, including man, mouse, and pig, have demonstrated that class I MHC antigens play an integral role in the cellular interaction leading to both humoral and cellular immunity. In

particular, the ability to accept tissue grafts appears to be determined by the class I MHC antigens. The ability to study the mechanisms regulating graft rejection and transplantation have been limited by the availability of appropriate pairs differing in only a single gene. The approach described here affords the possibility to study in vivo the effects of isolated genes on transplant rejection. An understanding of the genetic basis underlying transplantation rejection should allow for the development of new therapeutic modalities.

Proposed Course of Project: It should now be possible to determine the relation between those T cells specific for xenogeneic MHC products and the T cells specific for syngeneic and allogeneic murine MHC determinants. Specifically, the ability to generate CTL directed against the SLA antigen will be determined, as well as their precursor frequency and fine specificity, as determined using a panel of allogeneic and mutant cells. The mechanisms mediating murine self-tolerance to the normally foreign SLA antigens can be compared with those involved in conventional self-tolerance to murine H-2 products. Finally it will be of interest to determine the degree to which the murine T cell repertoire can utilize a transfected SLA gene product as a restricting element for antigen recognition and physiologic cell interactions.

Publications:

Frels, W., Bluestone, J., Hodes, R., Capecchi, M., and Singer, D.: 1985. Expression of a microinjected porcine class I MHC gene in transgenic mice. Science 228:580.

Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1984 to September 30, 1985

Members of the Laboratory of Immunobiology continued analyzing the effector arm of the immune system. Three major areas are under investigation: In the Humoral Immunity Section, analysis of the interaction of antigens, antibodies and complement components is the focus of activity; in the Immunopathology Section studies on chemotaxis and inflammation continued while in the Cellular Immunity Section the immunological basis of tumor rejection in vivo remained the central theme. There exists a close collaboration among the members of the Laboratory which made utilization of personnel effective.

Studies in the Humoral Immunity Section have continued to concern the binding, activation and action of antibodies and complement at cell surfaces. Last year we reported that complement activating ability of cell bound IgG and IgM antibodies depended on density and distribution of the hapten to which the Igs were bound. Most of these studies were performed with methotrexate and folinic acid as the haptens and rabbit and human Igs as the antibodies with the exception of an anti-methotrexate monoclonal antibody system. In collaboration with the Lausanne group (Professor Henri Isliker, principal collaborator) we have extensively studied the ability of mouse monoclonal antibodies to DNP to bind and activate complement. In a large series of experiments we have shown that the monoclonal antibodies to this hapten behaved essentially in the same manner as the rabbit polyclonal Igs and as the human IgM anti-methotrexate and anti-folinic acid antibodies: binding of C1 and activation of the C sequence were separable events activation being much more sensitive to small changes in hapten concentration than binding. Furthermore the efficiency of C activation by monoclonal antibodies was not significantly different from that of polyclonal antibodies provided there was a high concentration of hapten on the cell surface. In conjunction with these studies we also investigated the ability of IgG2b and IgM anti-DNP antibodies to generate the hemolytic complement intermediate EAC4 and the efficiency of this intermediate in measuring C1 activity; we found the there was no significant difference in the efficiency of these intermediates when compared to the efficiency of "conventional" cell intermediates. The results of these studies reinforced our conclusion reached during the past year that monoclonal antibodies behave very much like polyclonal antibodies under conditions where the epitope distribution is optimal.

In studying the dissociability of anti-hapten antibodies as a function of hapten density and fluid phase hapten concentration we have made the accidental observation that removal of the antibody from the cell surface by fluid phase hapten was greatly reduced by an anti-antibody. We have begun an extensive study of this phenomenon in collaboration with Dr. Rose Mage (NIAID). We have built up a collection of anti-allotype antibodies and anti-antibodies against various domains in the anti-hapten antibodies. Results so far indicate that dissociability of b4b4 or b4b5 anti-hapten antibody decreased at least by a factor of 10^5 when in combination with an anti-b4 antibody. In contrast, dissociability of the b4b5 anti-hapten antibody was barely affected by anti-b5 antibody. Antibodies to domains other than the allotype on the kappa chain had

different effects that varied from little or no effect to intermediate reduction in dissociability. The mechanism of the phenomenon is not understood at present but it cannot be ascribed to crosslinking of the anti-hapten antibodies by the anti-antibodies. The implication of this observation are far reaching because antibody-antibody complexes are highly C fixing and activating; if such a complex is attached to a cell surface and is essentially non-dissociable it has a greatly increased chance to induce complement mediated damage than it could induce in more dissociable form.

In continuing studies on the properties of complement sensitive and C resistant tumor cells attention was directed toward quantifying the number of C components, determining fatty acid positional analysis of selected phospho- and neutral lipids, and cell surface protein composition. Previous reports demonstrated that sensitivity of human, mouse and guinea pig tumor cells to C mediated killing could be enhanced by pretreating the cells in vitro with selected metabolic inhibitors whereas cells pretreated with selected hormones were resistant. Furthermore, in vitro grown human lymphoblastoid cells (Raji) and mouse mastocytoma cells (P815) were found to vary in their sensitivity to C killing at various stages of asynchronous growth. The Raji cells were resistant at stationary phase of growth whereas the P815 cells were resistant only at log phase growth. Extensive studies on lipid composition indicated that C sensitivity correlated with increased unsaturated fatty acids content.

The sensitivity of Raji and P815 cells could be manipulated by changing cell number or resuspending the cells in fresh culture medium. C resistant cells when suspended in fresh medium became sensitive to antibody C mediated attack within 15 minutes after incubation at 37°C but not at 40°C. No changes occurred in fatty acid composition or in fatty acid positional composition of isolated neutral phospholipids. Studies using ^{125}I labelling of cell surface proteins of the C sensitive and C resistant lines indicated that C sensitive Raji cells contained a 130K dalton protein not contained in the resistant Raji cells whereas C sensitive P815 lacked a 170K dalton and 16K dalton protein contained by the resistant P815 cells.

Studies have been initiated to determine if C resistance was due to lack of activation and binding of C components to the cells. Initial studies have been directed at binding of ^{125}I labelled purified human C3 to appropriately sensitized cells. These studies have shown that equivalent amounts of HuC3 were bound to the C sensitive and C resistant cells. In addition no differences were noted in the rate of C3 uptake by the antibody treated cells nor were there detectable differences in the loss of cell bound radioactivity. The results suggest that in addition to biochemical properties of the cell, binding, activation and interaction of the early acting C components with C3 and/or with the late acting C components and the cell membrane may play a role which is more important than appreciated thus far.

The Cellular Immunity Section has studied experimental tumors containing transplantation antigens encoded by well-characterized genes.

Biologic, immunologic, virologic and genetic studies were performed of retrovirus-infected fibrosarcomas. The properties of cloned and noncloned populations were examined. In agreement with the results of others, heterogeneity was a major feature of retroviral infection. Clones isolated from the mass infected culture differed in number of copies of the provirus

in cellular DNA, in release of infectious virus and in expression of retroviral antigens. After injection of the noncloned cell population into guinea pigs, tumor growth, regression and recurrence was observed. To better understand the mechanims by which tumors escape from immune attack, the properties of the tumor recurrences were determined. Previously we presented evidence that the tumor recurrences did not express retroviral cell surface antigens and were susceptible to superinfection with the homologuous retrovirus. In this work the major finding was that the cells that formed the tumor recurrences lacked the provirus. This conclusion was based on Southern blotting analysis of DNA extracted from recurrent tumors and recurrent tumor cell lines. Evidence indicates that the cells that form the recurrences have been selected from the infected cell population. Tumors formed in guinea pigs before the regression contain the provirus as do tumors formed in immunodepressed guinea pigs.

In studying the biology of this tumor, we developed a new method for tumor cell identification. Tumors lines were transfected with a bacterial gene (neo) that encodes resistance to the antibiotic G418. Colonies derived from cell lines were expanded and tested for the presence of the plasmid by Southern blotting. Each colony derived from a cell line contained a unique genetic marker. These unique genetic markers were stable in vivo. In addition to the unique genetic marker, cells were identifiable biochemically because the cells were resistant to the toxic effects of the antibiotic G418. These unique markers were used to determine whether tumor recurrences were of donor or recipient origin. Tumor cells transfected with the neo gene were superinfected with a retrovirus; tumor cells transfected with neo and infected with the retrovirus were injected into guinea pigs. Tumor recurrences were analyzed by Southern blotting for neo and for the provirus. The tumor recurrences retained the unique genetic markers of the parent transfected line and were able to proliferate in medium containing G418. The tumor recurrences lacked the provirus. This technique of tumor cell identification may be of general use in tumor biology.

Cells with partial, single or multiple copies of the provirus have been identified in the chronically-infected cell population. Cells with a single copy of the provirus might be at particularly high risk for genetic changes that would interfere with expression of retroviral cell surface antigens. One clone, A1, has been studied in detail. This clone contains an abbreviated copy of the provirus. Functional studies indicates that clone A1 contains a retroviral transplantation antigen and is resistant to infection with the homologous retrovirus. Genetic studies indicates that clone A1 contains the env of the provirus without other retroviral genes. During in vivo selection the retroviral transplantation antigen is lost.

These studies suggested that deletion of genes that encode cell surface antigens might be a mechanims of escape from immunological attack. An opportunty to explore this question was provided by an experimental tumor model developed by Dr. James Talmadge. A plasmid containing a gene ($H\text{-}2D^d$) for a murine major histocompatibility complex antigen was transfected into the B16 melanoma ($H\text{-}2^b$) cell line. This plasmid was stable during passage of the transfected cell line in vitro. Southern blotting analysis performed over several months on DNAs from transfected cell lines indicated that identical patterns were obtained after restriction with Eco RI and hybridization with a ^{32}P-labelled plasmid containing neo. The stability of the transfected plasmid was examined under conditions of selection in vivo. B16 melanoma cells tranfected with $H\text{-}2D^d$ were injected into syngeneic normal mice. The footpad tumors had the restriction

pattern characteristic of the cell line; the pulmonary metastases lacked either the part or the entire plasmid. This experimental tumor model appears to represent another example where deletion of a gene encoding a cell surface antigen is associated with escape from immunological attack. Studies are underway to attempt to prevent tumor escape from immunological attack.

Quantitative methods have been developed to measure the 9 components of rat complement. These assays have been used to monitor the changes that occur in rat plasma during absorption with Sepharose derivatives and to determine optimal conditions for consumption of complement components. The major finding was that C3 and C5 were consumed during absorption of rat plasma with Sepharose derivatives. The anticoagulant used to collect the blood had a major influence on C3 consumption. A series of *in vivo* experiments is in progress to analyze the effects of absorbed plasmas on the growth of primary mammary tumors.

In the Immunopathology Section the analysis of human monocyte heterogeneity and chemoattractant binding by flow cytometry was continued. The claim was confirmed that human monocytes and lymphocytes could be distinguished by different patterns of combined forward and 90 degree light scatter. However, when the number of monocytes within the monocyte light scatter window was compared with the input number of monocytes, it became apparent that not all monocytes were contained within the monocyte window. This analysis defined two groups of monocytes: core monocytes, those within the monocyte window; and outlier monocytes, outside the window. Outliers comprised a variable percentage of total monocytes, depending on the donor; the upper limit was approximately 40%. In a series of 7 experiments, 80 $\pm$ 2% (SEM) of core monocytes bound the chemoattractant fMet-Leu-Phe-Lys-FITC, whereas only 39 $\pm$ 12% of outlier monocytes bound the ligand. Chemoattractant binding of the whole blood monocyte population was compared with migration to chemoattractant. The percentage of all blood monocytes that bound fluoresceinated peptide attractant was 60 $\pm$ 8. In contrast, only 36 $\pm$ 3 (SEM for 16 experiments) percent of monocytes responded to attractant by directed migration. Thus, lack of chemotactic migration among human blood monocytes can be accounted for by absence of receptors among 1/3 of total monocytes and absence of directed migration despite receptors in an additional 1/3.

Although fMet-Leu-Phe is thought to be the prototype of chemoattractants produced by bacteria, there is no evidence that it is the major attractant in bacterial culture filtrates. During the year, a study of chemotactic factors in Staphylococcus aureus culture fluids was initiated. Chemotactic activity comprised a series of peptides that ranged from 600 to 2000 daltons. These peptides were resolved by reverse phase HPLC into 5 major peaks, one of which accounted for 40% of total activity. None of these peaks eluted in the location of fMet-Leu-Phe. Of great interest is the fact that in a series of 10 experiments, optimal concentrations of bacterial peptides attracted almost twice as many monocytes (53$\pm$5%) as the optimal concentration of fMet-Leu-Phe (30$\pm$3%). Studies are in progress to determine whether these differences are accounted for at the receptor level or subsequent to ligand-receptor interaction.

Several years ago, this laboratory discovered a mammalian serum protein that profoundly affected motility responses of mouse resident peritoneal macrophages. It was called macrophage stimulating protein (MSP), since it

caused macrophages in culture dishes to put out long cellular processes, and it made them responsive to chemoattractants. MSP has a molecular weight of 100,000; its isoelectric point shifts from 5.8 to 7.0 under mild conditions, suggesting a labile tertiary structure. The concentration in human serum is unknown, but is less than 100 ng/ml -- based on the lower limit of protein stain sensitivity in MSP-containing gels. Further purification of this trace protein would be facilitated with a monoclonal antibody. After failure of previous attempts to generate a monoclonal antibody, an improved approach has succeeded. Hybridoma well fluid was screened by adsorption of putative antibody to protein-A-Sepharose columns, after which a standard amount of MSP was applied to each column and residual MSP in the eluate was measured by bioassay. Depletion of eluate MSP reflected adsorption of MSP by the protein-A-anti-MSP complex. Maximal sensitivity was obtained by exposing the maximal amount of well fluid to gel and following with the minimal amount of MSP. This involved design and construction of simple, disposable, microliter immunoabsorbent columns. The columns accommodated 10 to 50 ul of gel; eluates were collected into capillary pipets. Because of minimal deadspace, all the eluate could be assayed. Four wells with anti-MSP were identified among 300 screened. One anti-MSP clone has been isolated, and IgG anti-MSP from mouse ascites fluid is now being collected.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08525-09 LIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunotherapy of Primary Autochthonous Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Zbar	Chief, Cellular Immunity Section	LIB NCI
	T. Borsos	Acting Chief, Laboratory of Immunobiology	LIB NCI
OTHER:	K. Nakanishi	Visiting Fellow	LIB NCI
	G. Glenn	Medical Staff Fellow	LIB NCI

COOPERATING UNITS *(If any)*

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI-FCRF, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	2.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The primary objective of this project is to evaluate biologic response modifiers in animals with primary cancer as a guide for treatment of human cancer.

GPO 914-918

Project Description

Objectives:

The primary objective of this project is to evaluate biologic response modifiers in animals with primary cancer as a guide for treatment of human cancer.

Methods Employed:

Breast cancers were induced in rats by a single intravenous injection of N-nitroso N-methyl urea into 50 day old animals.

Assays were developed to quantitate rat complement components on molecular basis.

Major Findings:

Reliable quantitative methods were developed to measure the 9 components of rat complement.

These methods were used to quantitate the changes that occur in rat complement during absorption with Sepharose derivatives. C3 and C5 activity was consumed during absorption under appropriate conditions with Sepharose 4B, inactivated CNBr Sepharose or protein A Sepharose. The concentration of functionally active C1 and C4 did not decrease during absorption with Sepharose derivatives.

C3 consumption in rat plasma anticoagulated with Acid Citrate Dextrose (ACD) was variable; addition of Mg++ (5 mM) to plasma anticoagulated with ACD augmented C3 consumption.

There was no C3 consumption in plasma anticoagulated with EDTA (a chelator of Ca++ and Mg++); C3 was consumed in plasma anticoagulated with EGTA (a chelator of Ca++).

The data suggests that Mg++ concentration limits C3 consumption in plasma anticoagulated with ACD.

The consumption of C3 without concomitant decrease in C1 or C4 and the requirements for Mg++ but not Ca++ are hallmarks of activation of the alternative pathway of complement.

Significance to Biomedical Research and Program of the Institute:

Previous work suggested that complement may be involved in the inhibition of tumor growth observed after infusion of plasmas absorbed with protein A. Terman et al. (J. Immunol., 124:795-805, 1980) reported a decrease in C3 antigen and deposition of C3 on tumor cell membranes 24 hours after plasma treatment. Gordon and coworkers (J. Natl. Cancer Inst., 70:1127-1133, 1983) noted that interaction of eluates of S. aureus with canine plasma consumed complement as measured by a reduction in CH50. Langone and associates (J. Immunol., 133:1057-1063, 1984) noted that interaction of Sepharose with human serum led to the generation of activated complement components C3a, C4a and C5a.

The significance of this work is that we have developed the tools to measure all 9 complement components of the rat. These newly developed methods will permit us to design in vivo experiments to evaluate the role of the complement system in the inhibition of growth of rat mammary carcinomas.

Proposed Course of Project:

Continuing efforts will be directed toward determining optimal conditions for plasma transfer therapy of rats with primary mammary carcinoma. Considerable variability in therapeutic results occur from experiment to experiment. The heterogeneity of growth of these primary mammary tumors is part of the problem. The oncogenes from tumors with different growth rates will be characterized. To eliminate variability associated with anticoagulation of blood, rats will be treated with serum obtained from tumor bearing animals. Rats will be treated by infusion of purified protein A or by infusion of plasma absorbed with formalin inactivated S. aureus cells.

Publications:

Nakanishi, K., Zbar, B., and Borsos, T.: Plasma Therapy of Primary Rat Mammary Carcinoma: Dependence of C3 Consumption during Absorption of Plasma with Sepharose Derivatives on the Anticoagulant. Cancer Res., in press.

Sukumar, S., Zbar, B., Terata, N., and Langone, J.J.: Plasma therapy of primary rat mammary carcinoma: antitumor activity of tumor-bearer plasma absorbed with inactivated CNBr Sepharose or protein A Sepharose. J. Biol. Resp. Mod., 3: 303-315, 1984

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08528-09 LIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanisms of Delayed Hypersensitivity and Tumor Graft Rejection

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Zbar	Chief, Cellular Immunity Section	LIB NCI
OTHER:	Y. Tanio	Visiting Fellow	LIB NCI

COOPERATING UNITS *(if any)*

A. Rein	Program Resources Inc., NCI-FCRF
J. Talmadge	Program Resources Inc., NCI-FCRF
B. McEwen	Upjohn Pharmaceutical, Kalamazoo, Michigan

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI-FCRF, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	1.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goals of this project are to analyze the genetic mechanisms by which immunogenic tumors escape host immune responses and the genetic basis of transplantation antigens of chemically-induced tumors.

Project Description

Objectives:

The goals of this project are to analyze the genetic mechanisms by which immunogenic tumors escape the host immune response and the genetic basis of transplantation antigens of chemically-induced tumors.

Methods Employed:

Strain 2 guinea pigs were obtained from the Experimental Animal Breeding Facility of the National Cancer Institute, Frederick Cancer Research Facility, Frederick, Maryland. Fibrosarcomas were obtained from Dr. Charles Evans, Laboratory of Biology, National Cancer Institute. Murine retroviruses MuLV 4070A and NFS Th-2 were obtained from Dr. Janet Hartley, National Institute of Allergy and Infectious Diseases. Viral proteins were detected with SDS-PAGE. Infectious virus was detected with the sarcoma positive, leukemia negative mink cell assay. The presence of the provirus was detected by Southern hybridization with a ^{32}P-labelled plasmid (AMT 8-1) containing the MuLV 4070A provirus.

B16 BL6 cell lines transfected with the Class I antigen D^d were obtained from Dr. James Talmadge, Program Resources Inc., NCI-FRCF. The presence of plasmid pGCneoD^d in genomic DNAs was detected by Southern blotting with ^{32}P-labelled pSVneo d.5.

Major Findings:

The major emphasis of the work has been on mechanisms of formation of tumor cell populations that have lost cell surface transplantation antigens. Antigen loss causes a profound change in the biology of the tumor-host relationship. When variants form tumors which grow and regress grow progressively leading to the death of the host. Antigen loss appears to be an irreversible change in tumor cell phenotype. Two models have been used in our work: retrovirus-infected fibrosarcoma cells and melanoma cells transfected with a gene encoding a Class I antigen.

Previously we reported that retrovirus-infected fibrosarcoma cells grew, regressed and then recurred in inbred guinea pigs. It is important to note that the cells tested were from cultures chronically-infected with retroviruses. Tumor recurrences lacked detectable viral proteins and were susceptible to infection with the homologous retrovirus.

This year we extracted genomic DNAs from tumor recurrences and demonstrated that the recurrences lack detectable proviral sequences. These studies indicate that the cells from a retrovirus-infected fibrosarcoma that survive *in vivo* immunologic selection lack the provirus.

We developed a method to determine if the tumors that arose at sites of regression were of donor or recipient origin. Colonies, isolated from fibrosarcoma cells transfected with neo, contained unique patterns of gene integration that were stable on transplantation in vivo. Tumors that recurred at sites of injection of marked retrovirus-infected cells contained the neo gene integrated in the form observed in cell lines.

Since these experiments were performed with heterogeneous cell population it was possible that the results could be explained by the presence of rare uninfected cells in the population. Experiments were repeated with a cloned nonproducer cell line containing a single abbreviated copy of the provirus. Immunological selection led to the formation of variants lacking the viral transplantation antigen. Preliminary results indicate that antigen loss variants from a cloned nonproducer line have lost the abbreviated copy of the provirus.

The most likely explanation for the formation of the recurrences is the deletion of proviral genetic information from fibrosarcoma cells. Cells with single copies of the provirus are thought to be at greater risk than cells with multiple copies of the provirus. Increasing proviral copy number by superinfection decreased the frequency of tumor recurrence.

Cell type had a major influence on the formation of tumor recurrences. The cell type, rather than the retrovirus, was the determining factor in the formation of tumor recurrences.

B16 BL6 cells ($H\text{-}2^b$) transfected with a gene encoding a Class I antigen (D^d) were injected into unimmunized mice or mice immunized to $H\text{-}2^d$ antigens. In unimmunized mice there was no difference in primary tumor growth of D^d transfectants compared to neo transfectants; survival after amputation of D^d tumors was prolonged. In immunized mice, primary tumor growth was retarded and survival was prolonged. Pulmonary metastases of both immunized and unimmunized mice and primary tumors of immunized mice had complete or partial loss of the transfected D^d plasmid. Antigen loss was associated with deletion of genes encoding cell surface antigens.

Significance to Biomedical Research and the Program of the Institute:

The major conclusion that emerges from this work is that tumors can escape from immunological attack by deletion of genes encoding cell surface transplantation antigens. In the immunocompetent host rare tumor cells that delete genes encoding cell surface antigens have a selective growth advantage and consequently may grow to form the predominant cell in the tumor. These events were observed both for a the retrovirus-infected fibrosarcoma and a melanoma transfected with a Class I antigen. The broader conclusion is that gene deletion in tumors may not be specific for loci containing retroviral or plasmid sequences. Gene deletion may be a more general event occurring randomly throughout the genome of the tumor cell. Gene deletion may be the one genetic basis for "dedifferentiation" of tumor cells.

Tumor lines were observed with characteristic distinct frequencies of recurrence after injection of retrovirus-infected cells. These differences in recurrence frequency might reflect differences in frequency of gene deletion in the various cell lines.

The method developed for identification of tumor cell in vivo may be generally applicable to a variety of problems in tumor biology. Transfection of neo into tumor cell lines provided both a biochemical marker (resistance to killing by G418) and unique genetic markers (unique restriction endonuclease patterns).

Proposed Course of the Project:

We will continue studies on antigen loss variants from nonproducer clone A1. The purpose of these studies will be to confirm results suggesting that antigen loss from clone A1 is associated with deletion of the provirus. Studies will be performed to study the influence of cell type on deletion of retroviral genes. A panel of cells is available with different frequencies of recurrence after retroviral infection. This panel will be probed with a number oncogenes to determine if there is a correlation between amplification of an oncogene(s) and retroviral gene deletion. Several molecular biologic methods will be used to isolate genes that encode antigens of chemically-induced tumors. Models will be developed to study the use of retroviral insertional inactivation as a method for isolation of genes that encode cell cell surface antigens. Monoclonal antibodies will be prepared to transplantation antigens of mutagen-treated tumor cells.

Publications:

Tanio, Y. and Zbar, B.: Immunogenicity and immunosensitivity of a guinea pig B-cell leukemia subline lacking cell surface Ia antigens. Cancer Res., 45: 143-148, 1985.

Terata, N., Tanio, Y., and Zbar, B.: A reliable method for reconstituting thymectomized lethally irradiated guinea pigs with bone marrow cells. J. Immunol. Methods, 70: 221-231, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08550-11 LIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Modification of Tumor Cells and Immune Cytolysis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: S. H. Ohanian Research Microbiologist LIB NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Humoral Immunity Section

INSTITUTE AND LOCATION

NCI-FCRF, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Pretreatment of guinea pig tumor cells with chemotherapeutic agents, metabolic inhibitors, enzymes or hormones modifies the susceptibility of the cells to killing by immune attack. Human and mouse tumor cells in asynchronous growth show variations in sensitivity to killing by antibody plus C. The purpose of this investigation is to determine the attributes of cells which influence the cells' ability to modify or resist cellular and humoral cytotoxic mechanisms.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

1) To study the mechanism whereby enzymes, metabolic inhibitors, anti-lipidemic agents and hormones affect the sensitivity of cells to immune attack; 2) to determine the metabolic pathways and physicochemical properties of the cell that may be modified following such treatment; 3) to determine the cellular processes that may be modified following or during immune attack, and 4) to determine the binding and activation of complement on the surfaces of C resistant and C sensitive cells.

Methods Employed:

Antibody is quantitated by the C1 fixation and transfer test, quantitative absorption tests, and immune cytolysis. Sensitivity of cells to antibody-C attack is measured by uptake of trypan blue and/or release of ^{125}IUdR. Incorporation of radioisotopically-labeled precursors of DNA, RNA, protein, complex carbohydrate, glycoproteins, glycolipids and lipids are being used to measure general metabolic properties of the cells. ^{125}I labeled C components are utilized to quantify the binding of C components. Thin layer chromatography is being utilized to analyze and identify specific lipid moieties synthesized by the cells. Sucrose density gradient ultracentrifugation is used to prepare plasma membrane and intracellular membrane fractions of tumor cells. High pressure liquid chromatography (HPLC) is being utilized to determine the lipid and fatty acid content and composition of the cells. SDS PAGE and HPLC are utilized to analyze protein and glycoprotein composition of membranes. Sensitivity of cells to cell-mediated immune attack is measured by ^{51}Cr release. Immunochemical methods, including Sephadex and DEAE chromatography, electrophoresis, immunodiffusion and ultracentrifugation are employed to isolate and identify biological macromolecules.

Major Findings:

Human lymphoblastoid cells, Raji and PY, and mouse mastocytoma, P815, show variations to antibody complement mediated killing during asynchronous growth in vitro. Raji cells were relatively more resistant to C killing during stationary phase of growth whereas P815 cells were relatively more resistant during log phase of growth. The difference in sensitivity of each cell line was not due to greater antigen expression or density on the C sensitive cells. In addition no correlation was noted between sensitivity to immune attack and synthesis of DNA, RNA, protein, complex carbohydrate and lipid. C sensitivity also could not be ascribed to fluid properties of the cells.

Previous studies with guinea pig hepatoma cells, mouse mastocytoma cells, and human lymphoid cells have demonstrated that sensitivity to C attack was dependent to some degree on lipid and fatty acid content of the cells. In general the content of selected unsaturated fatty acids was increased in the cells at the times the cells were sensitive to immune attack.

Further analysis of lipid synthesis of the human and mouse cells were performed using acetate and glycerol in addition to fatty acids. Cells incubated in fresh

media for 1 hr show a decrease in the incorporation of acetate and glycerol. In resistant Raji and P815 cells cultured in fresh media incorporation of unsaturated fatty acids was higher than that of saturated fatty acids. This appears to occur at the times the cells become sensitive to C killing. Although changes in total lipid composition do not occur changes in specific lipids (particularly for fatty acids, triglycerides, phosphatidylcholine and phosphatidylethanolamine) were noted. These results show that changes in lipid synthesis can occur very rapidly in cells and that different precursors of cellular macromolecules are used preferentially at one time but not another.

Analysis of lipid plasmalogen, prostaglandin synthesis (PGE_1 and PGF_1) and phospholipid methylation was performed with sensitive and resistant P815 and Raji cells. No differences were noted in total phospholipid plasmalogens of the resistant and sensitive cells. However, there is suggestive evidence that neutral lipid plasmalogens may be higher in sensitive cells. Preliminary studies on the cellular and extracellular concentration of PGE_1 and PGF_1 indicate no major differences between cells or media for resistant and sensitive populations. No correlation between phospholipid methylation and C sensitivity could be detected. Stationary phase Raji and P815 were both uniformly lower than log phase cells in the ability to methylate PE to PC. However, within 15 min after treatment of resistant cells with phospholipid methylation inhibitors, deazoadenosine plus homocysteine thiolactone or deoxycoformycin, homocysteine thiolactone plus adenosine, the cells became sensitive to C killing and the effect was reversible 3 hours after culture in medium free of inhibitor. Sensitive cells could also be made more sensitive following treatment with the phospholipid methylation inhibitors.

Studies using ^{125}I-labeled HuC3 to seed human C used to kill antibody sensitized cells indicated that no differences were detected in the amount C3 bound and the persistance of bound C3 for C sensitive and C resistant cells. Approximately 3 to 6 x 10^6 molecules of C3 were bound to the cells. In addition, all the C3 that was going to bind to the cells bound by 5 minutes after the addition of C. Further studies showed that the maximum number of C3 but not the rate of C3 binding to the cell was dependent upon the concentration of antibody used to sensitize the cell. No differences were noted in the kinetics of C3 binding to the C sensitive and C resistant cells. The results suggest that the relative resistance of nucleated cells to C killing is not due to the lack of binding or activation of C at least up to C3.

Significance to Biomedical Research and the Program of the Institute:

Modification of a tumor cell by metabolic inhibitors, hormones, chemotherapeutic agents and anti-lipidemic agents, or through chemical or physical manipulation of the cell's macromolecular composition furnishes a tool to study the interaction of tumor cells with the immune defense mechanisms of the host. The study of the response by tumor cells to humoral or cellular immune attack through modifications in various cellular metabolic pathways provides information regarding the mechanism of defense or cytomembrane repair processes in these cells. Modification of these processes may lead to cells that are more vulnerable to immune attack mechanisms. The availability of cell lines which show an innate variation to C killing also open up the possibility to study the processes which may be involved without investigator induced modifications.

Proposed Course of Project:

Radioisotope incorporation studies will be continued as probes to determine the cellular functions that are modified by treatment with drugs or hormones. The chemical attributes of normal cells, tumor cells and treated tumor cells will be studied. Work will continue on the effect of enzymes, hormones and inhibitors of macromolecular synthesis on the metabolic function of the tumor cells. Analysis of membrane-associated and intracellular macromolecules in these cells will be pursued. Further quantitative chemical analysis of the lipid and fatty acid and protein composition of cells that are susceptible or resistant to immune killing will be made.

The effect of antibody and C on nucleated cells will be further analyzed. This will include studies on the binding and utilization of C components during the cytotoxic reaction. The effects of the stages in the growth cycle of the cell and the physical and biochemical events that occur during formation and transformation of T* will also be studied. T* is an intermediate in the killing of cells by antibody plus C and contains all the components of C required for cell killing to occur.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08552-19 LIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanism of Complement Fixation and Action

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

PI:	T. Borsos	Chief, Humoral Immunity Section	LIB NCI
OTHER:	A. Circolo	Visiting Associate	LIB NCI
	P. Battista	Visiting Fellow	LIB NCI
	M. Kirschfink	Visiting Fellow	LIB NCI

COOPERATING UNITS *(if any)*

Department of Biochemistry, University of Lausanne

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Humoral Immunity Section

INSTITUTE AND LOCATION

NCI, NIH, FCRF, Frederick Md, 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.6	2.6	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

This is a long-range project investigating the mechanism of complement fixation and action. In particular the interaction of antibody-antigen complexes with the first component of complement and the result of this interaction on the other components are investigated. The relation between antibody action and complement activation is also explored. Finally, the significance of complement in the humoral immune defense mechanism is studied.

PHS 6040 (F. w. 1/84)

GPO 914-918

Project Description

Objectives:

To develop new methods and to use available complement (C) fixation tests based on the fixation and transfer of the first component of C (C1) and cytotoxic tests for the analysis of antigen-antibody reactions, in particular in the search for cancer specific antigens.

Methods Employed:

The model for studying cytotoxic reactions mediated by antibody and C consists of sheep erythrocytes, hemolytic antibody and C. Purification procedures for antibodies and the C components include: preparative (large-scale) gel filtration, ion exchange chromatography and preparative free electrophoresis. Other techniques used include precipitin and immunoelectrophoretic analysis, analytical, zonal and preparative ultracentrifugation and other immuno- and physico-chemical methods. Development and application of quantitative analytical methods which utilize radiolabeled tracer molecules are being emphasized.

Major findings:

We have extended our studies on the binding and activation of C1 and C by antibodies at cell surfaces to several mouse monoclonal antiDNP or TNP antibodies. In collaboration with the Lausanne group, we showed that the monoclonal anti-hapten antibodies bind and activate C very much like the anti-methotrexate rabbit antibodies, i.e. C1 binding can be dissociated from C activation by manipulating hapten density. In an extensive study we found that mouse IgM and IgG2b anti-DNP antibodies would generate the cell intermediate EAC4 with about the same efficiency as the rabbit polyclonal anti-Forssman IgM antibody. These findings indicate that monoclonal and polyclonal antibodies can be equally effective in inducing C activation provided hapten density is not the limiting factor.

An important variable in antibody action is its dissociability from its antigen (or hapten). In studying C binding and activation by antibody-anti-antibody complexes we made the accidental observation that cell bound anti-hapten antibody complexed with its anti-antibody was virtually none-dissociable by fluid phase hapten. The combination anti-b4 allotype antibody with a b4 anti-hapten antibody was particularly resistant to dissociation. Aggregation was most likely not the reason for this phenomenon for an anti-b5 antibody had little effect on the dissociability of a b5 anti-hapten antibody. Furthermore, aggregation of IgG anti-hapten antibody by protein A led to intermediate dissociability of the complex by fluid phase hapten.

Interaction of C1q with immuoglobulins may lead to the activation of the classical C pathway. It has been reported by others that fibronectin (FN) inhibits the actvity of C1q. We have analyzed the interaction of FN with cell bound Igs and C1q. We found that FN inhibited the uptake of C1q thereby apparently inhibiting C1q activity. When FN was added to C1q bound to Ig, we found no inhibiton of C1q activity leading to the suggestion that FN binds to C1q in a region of the molecule that is close to or is identical with the Ig

binding region. Thus FN may be a useful probe in studying the relation between activity and structure in the Clq molecule.

Significance to Biomedical Research and the Program of the Institute:

C fixation is one of the most widely used diagnostic tools. The development and successful application of a very sensitive C fixation test, the ClFT test, opened up new possibilities in determining antigen-antibody reactions on cell surfaces. Furthermore, cytotoxic reactions due to antibody and C are prime examples of body defense mechanisms. Fundamental research into the nature and mechanism of C fixation and action will contribute greatly to the development of diagnostic tools and to the understanding of the mechanism of immune body defenses.

Proposed Course of Project:

This is a long-range project, and little change is expected in the scope of the work during the next few years. The ultimate goals of this project are the development of better diagnostic tools and the elucidation of molecular events ass iate with the action of C and antibodies. It is hoped that as a result of ur program of inquiry into the basic problem of the interaction of antibodies, antigens and components of C, tools will be developed that are of practical significance in the search for cancer antigens and furnish a better understanding of the destruction of cells by antibodies and complement.

Publications:

Borsos T., and Circolo, A.: Binding and activation of hemolytic complement by IgG antibodies: cooperativity between antibodies of different hapten specificity. J. Immunol. 134: 492-494, 1985.

Kratz, H. J., Borsos, T., and Isliker, H.: Mouse monoclonal antibodies at the red cell surface. I. Generation of EAC4 and interaction with Cl. Molecular Immunol. 22: 223-227, 1985.

Kratz, H. J., Borsos, T., and Isliker, H.: Mouse monoclonal antibodies at the red cell surface. II. Effect of hapten density on complement fixation and activation. Molecular Immunol. 22: 229-235, 1985.

Circolo, A., Battista, P., and Borsos, T.: Efficiency of activation of complement by anti-hapten antibodies at the red cell surface: effect of patchy vs. random distribution of hapten. Molecular Immunol. 22: 207-214, 1985.

Battista, P., Circolo, A., and Borsos, T.: Activation of hemolytic complement by mouse monoclonal IgG1 antibody; comparison with rabbit IgG. Molecular Immunol. 22: 215-221, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08575-13 LIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Inflammation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. Leonard	Chief, Immunopathology Section	LIB NCI
OTHER:	Antal Rot	Visiting Fellow	LIB NCI

COOPERATING UNITS *(if any)*

	L. Henderson	Litton Bionetics Basic Research Program, FCRF

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Immunopathology Section

INSTITUTE AND LOCATION

NCI, NIH, FCRF, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Investigations in the Immunopathology Section are on chemotactic and other immune effector responses of leukocytes. The emphasis is on chemotaxis, a mechanism by which cells are attracted to inflammatory sites, delayed hypersensitivity reactions and growing tumors. The project includes chemistry and biology of bacterial derived chemotactic factors, characterization of a serum protein that modulates macrophage motility, and definition of functional subpopulations of blood monocytes.

PHS 6040 (Rev. 1/84) GPO 914-918

Major Findings:

Flow cytometry of human monocytes defined two subgroups of these cells, based on their light scatter properties. The claim was confirmed that human monocytes and lymphocytes could be distinguished by different patterns of combined forward and 90 degree light scatter. However, when the number of monocytes within the monocyte light scatter window was compared with the input number of monocytes, it became apparent that not all monocytes were contained within the monocyte window. This analysis defined two groups of monocytes: core monocytes, those within the monocyte window; and outlier monocytes, outside the window. Outliers comprised a variable percentage of total monocytes, depending on the donor; the upper limit was approximately 40%. In a series of 7 experiments, 80 $\pm$ 2% (SEM) of core monocytes bound the chemoattractant fMet-Leu-Phe-Lys-FITC, whereas only 39 $\pm$ 12% of outlier monocytes bound the ligand. Chemoattractant binding of the whole blood monocyte population was compared with migration to chemoattractant. The percentage of all blood monocytes that bound fluoresceinated peptide attractant was 60 $\pm$ 8. In contrast, only 36 $\pm$ 3 (SEM for 16 experiments) percent of monocytes responded to attractant by directed migration. Thus, lack of chemotactic migration among human blood monocytes can be accounted for by absence of receptors among 1/3 of total monocytes and absence of directed migration despite receptors in an additional 1/3.

A study of chemotactic factors in Staphylococcus aureus culture fluids was initiated during the current year. This was done because there is little evidence to support the presumption that fMet-Leu-Phe is the major chemoattractant produced by bacteria. S. aureus culture fluid chemotactic activity comprised a series of peptides that ranged from 600 to 2000 daltons. These peptides were resolved by reverse phase HPLC into 5 major peaks, one of which accounted for 40% of total activity. None of these peaks eluted in the location of fMet-Leu-Phe. Of great interest is the fact that in a series of 10 experiments, optimal concentrations of bacterial peptides attracted almost twice as many monocytes (53$\pm$5%) as the optimal concentration of fMet-Leu-Phe (30$\pm$3%). Thus, by both chemical and biological criteria, chemotactic activity of S. aureus culture fluid was not due to fMet-Leu-Phe. Studies are in progress to determine the basis for the high biological activity of the bacterial attractant.

Macrophage stimulating protein (MSP), a mammalian serum component that greatly enhances macrophage responsiveness to chemoattractants, was discovered in this laboratory. After failure of previous attempts to generate a monoclonal antibody to MSP, an improved approach has succeeded. Hybridoma well fluid was screened by adsorption of putative antibody to protein-A-Sepharose columns, after which a standard amount of MSP was applied to each column and residual MSP in the eluate was measured by bioassay. Depletion of eluate MSP reflected adsorption of MSP by the protein-A-anti-MSP complex. Maximal sensitivity was obtained by exposing the maximal amount of well fluid to gel and following with the minimal amount of MSP. This involved design and construction of simple, disposable, microliter immunoabsorbent

columns. The columns accommodated 10 to 50 ul of gel; eluates were collected into capillary pipets. Because of minimal deadspace, all the eluate could be assayed. Four wells with anti-MSP were identified among 300 screened. One anti-MSP clone has been isolated, and IgG anti-MSP from mouse ascites fluid is now being collected.

Significance to the Program of the Institute:

Tumor cell targeting by immune effector cells requires migration to tumor sites. Analysis of factors affecting motility and chemotaxis is important in understanding the host response and in devising immunotherapeutic approaches.

Proposed Course of Project:

Purification and characterization of monocyte chemoattractants produced by bacteria; isolation of macrophage stimulating protein (MSP) by affinity chromatography on a monoclonal anti-MSP column, development of an ELISA assay for the protein, establishment of normal serum concentration range and survey of human population for patients with values outside this range; further studies on functional properties of the two populations of human basophils described by this laboratory.

Publications:

James, S.L., Natovitz, P.C., Farrar, W.L. and Leonard, E.J. Macrophages as effector cells of protective immunity in murine schistosomiasis. VII. Macrophage activation in mice vaccinated with radiation-attenuated cercariae. Infection and Immunity 44:569-575,1984.

James, S.L., Correa-Oliveira, R., and Leonard, E.J. Defective vaccine-induced immunity to Schistosoma mansoni in P strain mice. II. Analysis of cellular responses. J. Immunol. 133: 1587-1593,1984.

Leonard, E.J., Skeel, A., and Alteri, E. Human monocyte chemotaxis: functional and flow cytometric analysis. Proceedings of the 10th International RES Congress, Ito, Japan, September 1984, in press.

Leonard, E.J. and Skeel, A. Separation of human basophils into two fractions with different density and histamine content. J. Allergy and Clinical Immunology, in press.

Leonard, E.J., Noer, K. and Skeel, A. Analysis of human monocyte chemoattractant binding by flow cytometry. J. Leukocyte Biology, in press.

Leonard, E.J. and Skeel, A. Disposable microliter immunoabsorbent columns: construction and operation. J. Immunol. Methods, in press.

Annual Report of the Laboratory of Cell Biology
National Cancer Institute
October 1, 1984 - September 30, 1985

The following are selected highlights of the research efforts of staff and collaborators of the Laboratory of Cell Biology.

Previous purification procedures for the TSTAs required the use of strong denaturing agents. However, to preserve native protein structure and biological activity, there has now been devised a mild ion-exchange chromatography step to prepare homogeneously pure TSTAs. These purified TSTAs retain their in vivo tumor rejecting activity and specificity when syngeneic hosts are immunized with the TSTA and then challenged with tumors from which the tumor antigens have been isolated (Meth A, CII-7 and mKSA). Biochemical characterization of the purified antigens show that the antigens are not glycoproteins and have molecular weights of 82,000 and 86,000, respectively, when analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). However, the apparent molecular weight of one of these antigens, p82, is approximately 68,000 when analyzed by other physiochemical methods. Under native conditions, p82 exists as a dimer or monomer and p86 only as a dimer. Partial sequence analysis of the NH_2- and COOH-termini of p82 indicates it represents a unique protein not previously described. The isoelectric point of p82 and p86 are 6.0 and 5.0, respectively. Sequence analysis of the p86 NH_2-terminal suggests the possibility that the two species may differ in their primary amino sequence. We are currently trying to determine if they indeed have different sequences and/or different post-translational modifications. p86 was also isolated from a virally-transformed sarcoma, mKSA and from the MC-induced sarcoma, CII-7 and was found to have the same pI as Meth A p86 and the same partial NH_2-terminal.

We hope to extend these sequence studies to find out if the individual TSTAs from a particular tumor have different primary sequences from that of their normal cellular counterparts or if the tumor rejecting activity is due solely to different subcellular distribution and/or other post-translational modification. This aim will be aided by the cloning of the p82 and p86 genes. We have synthesized several synthetic DNA probes, corresponding to partial protein sequences of the TSTAs, and they have been used successfully to screen a cDNA Meth A library. A partial sequence of one of the p82 positive clones has now been obtained. Both p82 and p86 are well conserved proteins and, by binding and precipitation assays, appear to be present on all tumors assayed. Although p82 and p86 are derived from cytosol, both are found on the cell surface by AB-peroxidase assays.

With the information from the protein and DNA sequence analysis and tissue distribution of the TSTAs, we hope to be able to elucidate the normal role of these proteins, the nature of their antigenic modification upon transformation and possible role in the maintenance and development of the transformed state. We believe that the changes in TSTAs are due to mutations affecting the primary sequence of p82 and p86 since it is hard to envision a post-translational modification which could generate unique TSTAs in each individual tumor.

Our studies have shown that the JB/RH (chemically induced), the K1735 (ultraviolet light induced), and the B16 (spontaneous), murine melanomas produce TSTA which not only display autologous tumor rejection activity, but share de-

terminants with the heterologous melanoma lines as well. Our specificity studies show that this activity is restricted to the melanoma cell lines tested, and there has been no cross-reaction with any of the non-melanoma tumor lines tested to date, with the exception of neuroblastoma cells (which have an identical embryologic derivation). Interestingly, the S91 (spontaneous) pigmented melanoma, although highly immunogenic, does not appear to share cell surface TSTA with other melanoma lines examined. Extraction of melanoma cells with aqueous butanol results in the quantative extraction of TSTA, and has proven to provide an excellent initial solubilization and purification step. The TSTA has now been further purified and has an apparent molecular weight of 50 to 80 kilodaltons; final purification of this molecule to homogeneity is in progress. The crossreacting TSTA found on the B16, the JB/RH and the K1735 melanomas are extremely interesting since these tumors were generated by radically different mechanisms, yet the TSTA do not display the typical unique specificity usually associated with TSTA from chemically induced and ultraviolet light induced neoplasms.

Membrane proteins from tranformed melanocytes vary in structure from those of normal melanocytes, and even from other types of transformed cells. It has been found that tumor-specific proteins released from melanoma cells can be found in the serologic fluids of tumor bearing patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro. It has been recently shown that one of these shed proteins has structural homology to the albumins, and may represent a normally occurring gene which is abnormally expressed in neoplastic tissues. Most recently, we have found that various subpopulations of melanoma cells growing in vivo after sequential transplantation become predominant, and that these differ in the structure of antigen expressed on the cell surface. Thus, TSA, isolated from sequential preparations of melanoma tumors, differed significantly in their amino terminal sequences, and this variability in antigen expression may be responsible for the diversity of subpopulations of melanoma cells that can be detected immunologically within a single tumor. In addition, the ability of this TSA (gp65), purified to homogeneity, to function as a TSTA has now been demonstrated. Thus, there are implications that these tumor-specific proteins may have critical immunologic importance to the survival of the tumor in the host.

Previous work in this laboratory has defined the T cell immune response specificities of different natural and synthetic cytochrome c peptides of mice with different H-2 haplotypes (i.e., I_a-restricted responses). T-cell clones specific for different cytochrome c peptides were established. These T-cell clones, along with antigen presenting cells, will be used in photochemical cross-linking experiments. Before these experiments could be carried out, a photochemical reagent specifically linked to the sulphydryl groups of the peptide and capable of being radio-iodinated was necessary. Since no cross-linking reagent with this specificity was available, it was necessary to synthesize this reagent. The requirement for sulphydryl coupling of the cross-linking reagent to the peptide was necessary since the alternative site, an α or ε amino group, is necessary for T-cell recognition by some of our clones. Synthesis has now been completed. The photo cross-linking reagent, azidosalicylic acid hexamethylene bromoacetamide, which alkylates free sulfhydryls, contains a photochemically reactive azido group and can be I-125 labelled to high specific activity. This reagent has been tested and preliminary experiments show it to be active only when irradiated with UV light. We are currently planning to use this reagent for antigen localization during the different stages of T-cell antigen recognition.

The receptor for antigen expressed by mouse helper and cytotoxic T lymphocytes is composed of a disulfide-linked dimer of about 85 Kd resolved under reducing conditions in two subunits (α and β chains) with an apparent M_r of about 42 Kd. Genes encoding the α and β chains of the T cell receptor for antigen are rearranged and expressed in helper and cytotoxic T cell clones. As to suppressor cells, human T cell clones with suppressive activity rearrange and express the β chain genes, whereas no somatic rearrangement of genes coding for the β chain has been observed in most mouse suppressor T cell hybridomas. We have described a cloned T cell lymphoma line, LH8-105, obtained by radiation leukemia virus-induced transformation of hen egg-white lysozyme (HEL)-specific mouse suppressor T lymphocytes. The LH8-105 suppressor T cell clone constitutively releases in the culture supernatant products able to specifically suppress the T cell-dependent proliferation, the antibody response and the delayed-type hypersensitivity to HEL. THis suppressive activity is restricted by genes apparently mapping to both the Igh-1 and I-J loci and is specific for a defined epitope in the N-terminal region of the HEL molecule. LH8-105 cells are Thy 1.2+, Lyt 2+, $I\text{-}J^+$ and they do not produce interleukin-2 nor gamma interferon and are not cytotoxic. They express on the cell surface dimers of 84 Kd composed of two 42 Kd subunits immunoprecipitated by rabbit antibodies recognizing T cell recptor structures. LH8-105 cells produce the β chain mRNA (1.3 Kb) expected for a functional T cell receptor and they delete both alleles of the $C\beta_1$ gene and rearrange both alleles of the $C\beta_2$ gene, as detected by a Cβ probe from helper T cell hybridoma. Moreover, LH8-105 cells rearrange and express genes encoding the α chain of the T cell receptor. These findings indicate that at least some mouse suppressor T cells use the same set of genes as helper and cytotoxic T cells for their antigen-specific membrane receptor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03200-16 LCBGY

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Factors Influencing the Induction, Growth and Repression of Neoplasms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. W. Law	Chief, Lab. of Cell Biology	LCB, NCI
OTHER:	E. Appella	Medical Officer (Res.)	LCB, NCI
	V. J. Hearing, Jr.	Res. Biologist	LCB, NCI
	E. A. Robinson	Chemist	LCB, NCI
	S. J. Ullrich	Sr. Staff Fellow	LCB, NCI

COOPERATING UNITS *(if any)*

Sloan-Kettering Cancer Center, New York, NY
Yale University, New Haven, CT
LG, DCBD, NCI

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	1	3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Major emphasis is placed upon the study of Class I and Class II tumor antigens of the transplantation rejection type (TATA), and of tumor antigens (TA) assayed by in vitro techniques and of the immune responses they evoke. As a corollary to this study the biologic properties in vitro and in vivo of alien histocompatibility (H-2) antigens and of variant antigens in several neoplasms are under study. Solubilization and methods of purification of TATAs are under investigation with the ultimate purpose of defining these membrane and cytosol antigens, after purification, in physicochemical, biologic and molecular terms.

Other Professional Personnel:

M. Rogers	Biochemist	LG NCI
A. DeLeo	Staff Member	Sloan-Kettering Cancer Ctr.
G. DuBois	Biochemist	FCRF
A. LeClair	Staff Member	Yale University

Project Description

Objectives:

To characterize tumor antigens, principally those having tumor rejection activity (TATA) of different origins and etiologies (chemically-induced tumors, melanomas, leukemias). To isolate, purify and characterize the immunogenic properties of these TATA and define their molecular basis and genetic identity. To attempt to relate these findings to the process of oncogenesis and for diagnosis and therapy of cancer.

Methods employed:

Various chemical, biochemical and immunologic techniques are employed such as: development of appropriate in vivo models that lend themselves to adequate immunogenecity assays that define specificity both in vivo and in vitro, development of methods for isolation and purification of tumor antigens; each antigen has peculiar characteristics such as lability, sensitivity to proteases, behavior on gels etc., development of appropriate tumor specific antisera, using purified proteins so that this a long, arduous task.

Major findings:

TUMOR ANTIGENS:

1. Studies of Isolation and Purification of Unique Tumor Rejection Antigens (TATA)

A. The TATA of the chemically induced sarcomas Meth A and CI-4 were purified to apparent chemical homogeneity from the cytosol of dissociated cells (DuBois, Appella and Law, LCB). These antigens were purified by preparative electrophoresis in the presence of sodium dodecyl sulfate or by immunoaffinity chromatography following hexylamine agarose, gel filtration (Sephacryl S300) and hydroxylapatite chromatography. Although our earlier study identified the TATAs of both sarcomas as a 75 kDal protein, subsequent more extensive characterization from several sarcomas leads to the conclusion that these TATA are approximately 82 kDal, presenting as separate bands on SDS-PAGE. Although both Meth A and CI-4 antigens have approximately the same MWs, they differ in their chromatographic behaviors on hexylamine agarose and hydroxylapatite. Each purified TATA, however, retains its individual specificity as the tumor of origin and each is immunogenic

at low µg levels.

B. Recently we have isolated and purified a tumor rejection antigen from an SV40-induced sarcoma, mKSA of BALB/c mice. The purification method employed in this study was essentially the same as that used to isolate and purify TATA from Meth A although a final mild ion-exchange chromatograph step (FPLC) is now used (see report of S. Ullrich for details). This TATA was tentatively identified as an 86 kDal protein on 8-10% acrylamide gels and presented apparently as a single band on SDS-PAGE. This newly isolated TATA was found to be immunogenic at 10-30 µg levels specifically against mKSA sarcoma challenge. It did not protect against Meth A, CI-4 and CII-10 challenge and interestingly did not protect against VLM challenge; VLM is also an SV40-induced sarcoma in BALB/c mice that normally crossreacts with mKSA since both share the large SV40 T antigen. We used an ELISA for detection of any SV40 antigen or components in our 86 kDal protein. Anti-SV40 T antigen antiserum detected SV40 on SV80 (malignant cells) and Vero cells infected with SV40 but not in the 86 kDal preparation. Thus, this isolated antigen that exists on mKSA sarcoma cells resembles, in many characteristics, the TATA isolated from chemically induced sarcomas and its specific tumor rejection function is seen only after removal of the immunodominant SV40 group specific antigen.

In further isolation and purification of this prominent and unique TATA of mKSA, on polyacrylamide gel electrophoresis, Dr. Stephen Ullrich (this laboratory) has resolved this so-called 86 kDal protein into two distinct bands of approximately 83 kDal and 79 kDal molecular weights. These, however, have not been adequately separated as yet and assayed for immunogenicity. Proteins prepared and isolated in the same manner from Meth A sarcoma cells also contain fractions that elute from hydroxylapatite at the same salt concentrations and upon SDS-PAGE show the same two protein bands at 83 kDal and 79 kDal. These are quite separate from the originally identified 82 kDal protein. p86 has now been purified from Meth A (in addition to p82) and from a newly arisen MCA-induced sarcoma, CII-7 (in collaboration with G. DuBois (FCRF). Each p86 retains its specificity for the tumor from which it was isolated (mKSA, Meth A and CII-7) as determined by in vivo tumor rejection assays. These separate p86 antigens have the same PIs, MWs and, as far as accomplished, identical NH_2 terminal amino acid sequences (for mKSA and Meth A) according to E. Robinson.

Partial sequence analyses from both NH_2- and COOH- termini for p82 have now been obtained (see Robinson and Appella). p82 appears to be a unique protein not previously described (from the Computer Data Bank). Preliminary NH_2 terminal sequencing has also been done for p86 from both Meth A and mKSA and it appears that p82 and p86 are separate species.

p82 and p86-like proteins have been obtained through the use of rabbit antibodies made against the purified proteins (α p82 and α p86) by ^{35}S-methionine precipitation (G. DuBois) in most tumors and normal tissues studied. I^{125}-binding assays (K. Tanaka) parallel the precipitation findings. Thus, p82 and p86 appear to be well-conserved proteins.

Although p82 and p86 have been isolated and purified from the cytosol, Avidin-Biotin-Peroxidase assays (Shu, Pathology Branch) show a distribution of both antigens on the cell surface (at least for Meth A sarcoma cells)

2. Studies of Melanoma-associated (common) Antigens

Collaborative work continues with V. Hearing in characterizing, immunologically and biochemically, the tumor rejection antigens (TATA) of a group of

murine melanomas of different origins: B-16 (F10), K1735, JB/RH and S91 (see report of V. Hearing). The interesting findings, to date, are as follows:

a. Each of the melanomas is immunogenic in syngeneic mice,

b. In contrast to the nature of TATA from chemically-induced tumors, these melanomas show a crossreactive pattern among themselves but apparently specific for the melanomas. Thus these tumor antigens (Class II) are tissue specific in contrast to the absolute restriction shown by UV-induced or chemically-induced sarcomas (Class I). No crossreactivity was observed for the melanoma TATA with any other tumor.

c. The presence of common TATA among the melanomas, however, is not absolute since preliminary findings indicate that S-91, a highly immunogenic melanoma, appears not to crossreact with K1735, B-16 and JB/RH.

d. K1735 and JB/RH behave similarly to human melanomas in releasing their antigens spontaneously or being easily extracted by 2% butanol. The immunogenic and crossreactive characteristics of the soluble TATA thus released parallel the findings with intact cells in quantitative studies of in vivo assays.

e. The TATA of one of the melanomas, B-16, has been purified. In contrast to the antigens of methylcholanthrene tumors, it is a glycoprotein. It has a molecular weight of 65,000 daltons and an isoelectric point of 4.5. In previous studies (Marchalonis, Hearing, Gerston), this gp65 had a significant sequence homology to albumin. It has now been shown that gp65 effectively immunizes against challenge with all of the murine melanomas assayed but not against non-melanoma tumors. It also strikingly inhibits the lung colonization of B-16 melanoma cells. Progress is now being made in purifying gp65 from the K1735 and JB/RH melanomas.

The tumor antigens we have identified appear to be entities distinct from the antigens studied on human melanomas through the use of monoclonal antibodies.

3. Studies of Leukemia Antigens

Previous collaborative work with M. Rogers and colleagues (LG) led to the identification, isolation and purification of a TATA from the T-cell lymphoma RBL-5 on a 175 kDal molecule, a glycoprotein. This antigen, in nanogram amounts, completed suppressed RBL-5 and other FMR-induced leukemias, but no other tumors tested. Recently a second TATA-bearing molecule that does not crossreact serologically with gp175, or with viral structural proteins, has been identified and partially purified from RBL-5 (G. Galetto). Like gp175, this TATA, gp50, is immunogenic against FMR leukemias (see Annual Report of M. Rogers).

4. Continued Phenotypic Studies of BALB/3T3 Cells Transfected with DNA from MC- induced neoplasms

The ability of DNA from chemically tranformed cells to transfer the malignant phenotype to non-transformed cells (3T3/BALB) provides a method for examining the relationship between TATA expression and cellular transformation. The stable expression of the unique TATA by most chemically induced sarcomas is indicative of a close coupling of the events involved in transformation and expression of TATA. In our initial studies with Hopkins (MIT) and DeLeo (Sloan-Kettering) although we found a high frequency of co-expression of the transformation in phenotype and Meth A expression (both serologically and by tumor rejection), it was found in cloning experiments, by Hopkins, that the Meth A used for preparation of DNA contained a polyoma virus sequence capable of transforming

3T3 cells at a high frequency. Extensive studies showed that the small polyoma sequence had no relationship, however, to the Meth A TATA.

We have now repeated the DNA transfection studies in collaboration with K. P. LeClair (Yale University) and A. B. DeLeo (Sloan-Kettering) using DNA from the parental Meth A line that is free of any polyoma sequences. Again we have found, in two of three cloned transfectants, expression of the unique Meth A TATA as determined by tumor rejections and A. DeLeo has identified the Meth A antigen using serologic probes in the same transfectants. Since the transforming element of some MC-induced tumors appears to be the Kirsten ras protein, it is possible that ras gene probes may be used in the isolation of the Meth A gene. LeClair has not yet completed his survey of these transfectants.

Recently we studied (in collaboration with Dr. A. Eva) a series of 3T3/NIH primary and secondary transfectants obtained from using the DNA of C57Bl/6 and NIH Swiss chemically-induced sarcomas in which C-kis has been recognized as the transforming gene. (See Eva and Aaronson, Science 220, 527, 1983). These studies were inconclusive. Two of the transfectants from sarcoma T-92497 were not capable of immunizing against the parental sarcoma although T-92497 had an easily identifiable TATA. Sarcoma MCA-IGP12 did not have an identifiable TATA, nor did the two transfectants obtained from this sarcoma.

Significance:

Characterization of tumor antigens of the rejection type is a necessary prerequisite for understanding the mechanisms of immune surveillance, tumor inhibition and facilitation. In addition, the role of these cytosol and membrane components in the mechanisms of initiation and maintenance of malignancy will be studied.

As a basis for any study of membrane-bound antigens, it is necessary to study the nature of histocompatibility antigens and their relationship to tumor antigens. Thus our emphasis is on parallel studies of H-2 antigens in order to provide a basis for understanding tumor antigens.

Proposed Course of Research:

See section on Major Findings.

Publications:

DuBois, G.C., Appella, E., and Law, L.W.: Isolation of a tumor-associated transplantation antigen (TATA) from an SV40-induced sarcoma. Resemblance to the TATA of chemically induced neoplasms. Int. J. Cancer 34: 561-566, 1984.

Law, L.W.: Assignment of the gene encoding for Meth A tumor rejection antigen (TATA) to chromosome 12 of the mouse. Brit. J. Cancer 50: 104-111, 1984.
Hearing, V.J., Vieira, W.D, and Law, L.W.: Malignant Melanoma: Cross-reacting (common) tumor rejection antigens. Int. J. Cancer 35: 403-409, 1985.

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Law, L.W.: Characteristics of tumor antigens on tumors induced by chemical carcinogens. Some recent findings. In Reif, A. and Mitchel, M. (Eds.): Proceedings Symposium Immunity and Cancer. Academic Press (in press).

Galetto, G., Law, L.W., and Rogers, M.: The Rauscher-MuLV induced leukemia, RBL-5 bears two tumor associated transplantation antigens expressed on distinct molecules. Int. J. Cancer (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03229-16 LCBGY

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structural Analysis of Histocompatibility and Tumor Antigens and T-cell Receptors

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. Appella	Medical Officer (Res.)	LCB, NCI
OTHER:	E. Robinson	Chemist	LCB, NCI
	S. Ullrich	Sr. Staff Fellow	LCB, NCI
	R. De Santis	Visiting Fellow	LCB, NCI

COOPERATING UNITS *(if any)*
Fox Chase Cancer Center, Philadelphia, PA
Weizmann Institute of Sciences, Rehovot, Israel
Lab. of Development & Mol. Immunol., NICHD
Lab. of Cell Biol., NHLBI

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Chemistry

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD

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6	3	2

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

We are studying the molecular structure of histocompatibility antigens, tumor antigens, and T-cell receptors. A combination of protein and DNA sequencing, in conjunction with peptide and nucleotide synthesis, is being used in order to reach a better understanding of the molecular architecture of these important biological molecules. Oligonucleotide directed site mutagenesis has been employed to elucidate the role of individual amino acids on the function and expression of histocompatibility class I and II antigens. Synthetic peptides corresponding to oncogene structures such as myc and ras are being generated and antibodies are being made to study the role that these proteins play in transformation and in normal growth control. Homogeneous DNA sequences predicted to form aberrant helices with inserted bases and hairpin stem and loops are being crystallized. These are model structures that will help clarify why certain sequences have a much greater tendency to mutate than others.

GPO 914-918

Other Professional Personnel:

D. Hansburg	Research Biologist	Fox Chase Cancer Center
E. Korn	Chief	LCB NHLBI
K. Ozato	Immunochemist	LI LCIHD
J. Sussman	Professor	Weizmann Institute
S. Weinstein	Guest Researcher	Weizmann Institute
M. Willingham	Research Biologist	LMB NCI

Project Description

Objectives:

Structural analysis of histocompabibility antigens, tumor antigens and T-cell receptors. Isolation and characterization of oncogene proteins such as myc and ras. Analysis of structures like myosin which will help to understand the mechanisms involved in phosphorylation of particular serine residues and the modulation of enzymatic activity. Crystallization of particular DNA structures designed to elucidate the molecular mechanism of genetic regulation and mutation.

Methods employed:

Biochemical techniques such as hydrophobic and ion exchange chromatography, isoelectric focusing and HPLC were used for isolation and purification of proteins. Gas-liquid microsequencing analysis of protein and peptides. Nucleic acid sequencing of genes by chemical cleavage or dideoxy methods. Solid-phase synthesis of peptides and nucleic acid oligomers.

Major findings:

HISTOCOMPATIBILITY ANTIGENS

The mouse histocompatibility complex (MHC) plays a major role in the regulation of the immune response. The class I cell membrane associated molecules (K, D and L), or transplantation antigens, have been shown to be present on all somatic cells. The K and D loci have more than 50 serologically distinct alleles. We have completed the entire nucleotide sequence of the H-2D^{p} class I gene. The H-2D^{p} nucleotide sequence is greater than 90% homologous to the H-2L^{d} and H-2D^{b} genes and only 85% to the H-2D^{d} gene. The K and Qa regions are less homologous. All D-region genes, D^{b}, L^{d} and D^{d}, possess the ALU-like repetitive sequence as does D^{p}. This sequence is a marker present in all D-region genes so far studied. An additional polyadenylation site was found in the H-2DP gene, which is present only in non-D-region sequences. This characteristic makes the H-2DP gene an interesting model for studying the evolution of polymorphism and structure/function relationships in the class I gene family.

Forty-five new monoclonal antibodies reacting with the mouse H-2D^{d} antigen have been established. The specificities of 34 of these antibodies were mapped to the first external domain (N) of the D^{d} antigen. All but two of the allospecificities corresponded to either one or multiple positions of amino acid substitutions in the N domain. Because allodeterminants most likely signify the

sites for H-2 restriction, mapping by site-specific in vitro mutagenesis of such determinants is being carried out to shed some light on the structural basis for T cell reactivity.

T CELL ANTIGEN IMMUNE RESPONSE

The receptor for antigen expressed by mouse helper and cytotoxic T lymphocytes is composed of a disulfide-linked dimer of about 85 Kd resolved under reducing conditions in two subunits (α and β chains) with an apparent M_r of about 42 Kd. Genes encoding the α and β chains of the T cell receptor for antigen are rearranged and expressed in helper and cytotoxic T cell clones. As to suppressor cells, human T cell clones with suppressive activity rearrange and express the β chain genes, whereas no somatic rearrangement of genes coding for the β chain has been observed in most mouse suppressor T cell hybridomas.

We have described a cloned T cell lymphoma line, LH8-105 obtained by radiation leukemia virus-induced transformation of hen egg-white lysozyme (HEL)-specific mouse suppressor T lymphocytes. The LH8-105 suppressor T cell clone constitutively releases in the culture supernatant products able to specifically suppress the T cell-dependent proliferation, the antibody response and the delayed-type hypersensitivity to HEL. This suppressive activity is restricted by genes apparently mapping to both the Igh-1 and I-J loci and is specific for a defined epitope in the N-terminal region of the HEL molecule. LH8-105 cells are Thy 1.2+, Lyt 2+, $I\text{-}J^+$ and they do not produce interleukin-2 nor gamma interferon and are not cytotoxic. They express on the cell surface dimers of 84 Kd composed of two 42 Kd subunits immunoprecipitated by rabbit antibodies recognizing T cell receptor structures. LH8-105 cells produce the β chain mRNA (1.3 Kb) expected for a functional T cell receptor and they delete both alleles of the $C\beta_1$ gene and rearrange both alleles of the $C\beta_2$ gene, as detected by a Cβ probe from helper T cell hybridoma. Moreover, LH8-105 cells rearrange and express genes encoding the α chain of the T cell receptor. These findings indicate that at least some mouse suppressor T cells use the same set of genes as helper and cytotoxic T cells for their antigen-specific membrane receptor.

Previous work in this laboratory with Dr. Daniel Hansburg has defined the T cell immune-response specificity of different natural and synthetic cytochrome c peptides in mice with different H-2 haplotypes (i.e., Ia-restricted responses). Cell clones specific for different cytochrome c peptides were established. These T-cell clones, along with antigen presenting cells, will be used in photochemical cross-linking experiments. Before these experiments could be carried out, a photochemical reagent specifically linked to the sulphydryl groups of the peptide and capable of being radioiodinated was necessary. Since no cross-linking reagent with this specificity was available, it was necessary to synthesize this reagent. The requirement for sulphydryl coupling of the cross-linking reagent to the peptide was necessary since the alternative site, amino groups, are necessary for T-cell recognition by some of our clones. Therefore, Dr. Shulamith Weinstein synthesized the photo cross-linking reagent, azidosalicylic acid hexamethylene bromoacetamide, which alkylates free sulfhydryls, contains a photochemically reactive azido group and can be I-125 labelled to high specific activity. This reagent has been tested and preliminary experiments show it to be active only when irradiated with UV light. We are currently planning to use this reagent for antigen localization during the different stages of T-cell antigen recognition.

TUMOR SPECIFIC TRANSPLANTATION ANTIGENS

Previous purification procedures for the TSTAs from Meth A tumors required the use of denaturing agents. However, to preserve native protein structure and biological activity, we have devised a mild ion-exchange chromatography step to prepare homogeneously pure TSTAs. These purified TSTAs retain their in vivo tumor rejecting activity when syngeneic hosts are immunized with the TSTA and then challenged with Meth A tumors but not other related tumors. Biochemical characterization of the purified antigens shows that the antigens are not glycoproteins and have molecular weights of 82,000 and 86,000 when analyzed by SDS polyacrylamide gel electrophoresis. However, the apparent molecular weight of p82 is approximately 70,000 when analyzed by other physicochemical methods. Under native conditions, p82 exists as a dimer or monomer and p86 only as a dimer. Partial sequence analysis of the NH_2- and COOH-termini of p82 indicates it represents a unique protein not previously described. The isoelectric points of p82 and p86 are 6.0 and 5.0, respectively, in both Meth A and NIH 3T3 cells. The p86 antigen actually represents two molecular species of very similar size and charge. Sequence analysis of the p86 NH_2-terminal indicates that the two species differ in their amino acid sequences in several, but not all, positions. p86 was also isolated from a virally-transformed sarcoma, mKSA, and was found to have the same pI as Meth A p86 and the same partial NH_2-terminal sequences.

We hope to extend these sequence studies to find out if the individual TSTAs from a particular tumor have different primary sequences from that of their normal cellular counterparts or if the tumor rejecting activity is due solely to different subcellular distribution and/or other post-translational modification. This aim will be aided by the cloning of the p82 and p86 genes. We have synthesized several synthetic DNA probes, corresponding to partial protein sequences of the TSTAs, and they have been used successfully to screen a mouse cDNA library. A partial sequence of one of the p82 positive clones has now been obtained.

Immunofluorescent studies of p82 and p86 were performed to study the subcellular localization and tissue distribution of the TSTAs. The results demonstrate that both TSTAs are normally cytosolic proteins but, upon transformation, they are also present at the cell surface. Immune-electron microscopy of the TSTA shows that they don't appear to have discreet cytoplasmic locations but are present throughout the cytoplasm. Furthermore, immunofluoresence indicates that there is more p86 compared to p82 present in Meth A cells. A tissue survey of p82 and p86 in normal mouse tissues, using fixed sections and anti-TSTA sera showed that p86 is present in high concentrations in the cytoplasm of oocytes, and in the apical region of the uterus, bladder and small intestinal epithelial cells. p82 was apparently either not expressed in sufficient amounts to be detected or was not preserved by the fixation method employed. Therefore, frozen sections of mouse tissued will be used to examine its tissue distribution.

With the information from the protein and DNA sequence analysis and tissue distribution of the TSTAs, we hope to be able to elucidate the normal role of these proteins, the nature of their antigenic modification upon transformation and theif possible role in the maintenance and development of the transformed state. We believe that the changes in TSTAs are due to mutations affecting the primary sequence of p82 and p86 since it is hard to envision a post-translational modification which could generate unique TSTAs in each individual tumor.

PROTEIN AND NUCLEIC ACID CHEMISTRY

Myosin II from Acanthamoeba castellani has an actin-activated Mg^{+2}-ATPase activity which is regulated by phosphorylation of three serine residues near the carboxyl terminus of the molecule. We have synthesized four peptides in this region and used these to raise polyclonal antibodies. Using these reagents, we have probed this region of the molecule to investigate how it might control filament assembly and conformation. Our results indicate that the enzymatic activity is sensitive to the filament conformation and that this is regulated through the interaction of the terminal regions of the coiled-coil part of the tails of individual myosin molecules.

During the past few years, there has been growing evidence that the structure of DNA may be even more complex than previously thought. Of great importance are the strong arguments that aberrant DNA structures are the precursors to genetic mutation. Detailed structural information is required to confirm that these structures do exist and to elucidate the molecular mechanism of genetic regulation and mutation. We have started to synthesize, crystallize and determine the three dimensional structure by X-ray crystallography of homogenous DNA sequences predicted to form structures with deviations from the classical double helix. We have already obtained crystals from the sequence 5'CGCAGAATTCGCG3'. This sequence is a model structure for what most likely is a precursor to frameshift mutations.

Significance to biomedical research:

These studies are aimed to an understanding of the relationship of structure to the function of macromolecules involved in the immune system. The T-cell receptor is a molecule whose function is central to our understanding of cellular immunity. Furthermore, its relationship to the restricting element represented by class I or II histocompatibility antigens is of equal importance in order to understand T-cell function. The elucidation of the structure of tumor antigens of the transplantation type has long been a goal of tumor immunologists. Our approach should resolve the question of the structure-function of these particular antigens in comparison to the established response of normal T-cell antigens. The cytoskeleton is an important structure for both normal and transformed cells. The studies of non-muscle myosin should shed some light on the mechanism involved in the interaction of this protein with other structures. The studies of DNA model structures of likely precursors to frameshift mutations will help to clarify the molecular mechanism of genetic regulation and mutation.

Proposed course of research:

The studies as outlined are either completed or currently in progress.

Publications:

Appella, E., and Sawicki, J.A.: β2-microglobulin. In Di Sabato, G., Langone, J. J., and Van Vunakis, H. (Eds.): Methods in Enzymology, Immunochemical Tech-Techniques San Diego, Academic Press, 1984, pp. 494-504.

DuBois, G.C., Appella, E., and Law, L.W.: Isolation of a tumor-associated transplantation antigen (TATA) from an SV40-induced sarcoma. Resemblance to the TATA of chemically induced neoplasms. Int. J. Cancer 34: 561-566, 1984.

Shiroishi, T., Evans, G.A., Appella, E., and Ozato, K.: Role of disulfide bridge in the immune function of major histocompatibility class I antigen as studied by in vitro mutagenesis. Proc. Natl. Acad. Sci. 81: 7544-7548, 1984.

Cote, G.P., Robinson, E.A., Appella, E., and Korn, E.D.: Amino acid sequence of a segment of the acanthamoeba myosin II heavy chain containing all three regulatory phosphorylation sites. J. Biol. Chem. 259: 12781-12787, 1984.

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Ozato, K., Takahashi, H., Appella, E., Sears, D.W., Murre, C., Seidman, J.G., Kimura, S., and Tada, N.: Polymorphism of murine major histocompatibility class I antigen. J. Immunol. 134: 1749-1758, 1985.

Adorini, L., Colizzi, V., Pini, C., Appella, E., and Doria, G.: Immunoregulation of lysozyme-specific suppression: Analysis of the efferent phase in the suppressive circuit induced by hen egg-while lysozyme specific monoclonal suppressor T cell products. J. Immunol. (in press).

Saper, M., Eldar, H., Appella, E., and Sussman, J.L.: Crystallization of a DNA tridecamer CGCAGAATTCGCG. J. Mol. Biol. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09100-2 LCBGY

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunogenicity of Melanoma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Vincent J. Hearing, Jr.	Research Biologist	LCB, NCI
OTHER:	Lloyd W. Law	Chief, Lab. of Cell Biology	LCB, NCI
	Ettore Appella	Medical Officer (Res.)	LCB, NCI

COOPERATING UNITS *(if any)*

Intl. Inst of Gen. & Biophysics, Milan, Italy
Tohoku Univ. School of Med., Sendai, Japan
Georgetown Univ. School of Med., Washington, D.C.
Med. Univ. of SC, Charleston, SC
Regina Elena Cancer Inst., Rome
Johns Hopkins Univ., Baltimore

LAB/BRANCH

Laboratory of Cell Biology

SECTION

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NCI, NIH, Bethesda, MD 20205

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2.5	1.2	1.3

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project is aimed at elucidating the host immune response to malignant melanoma, and the role this response plays in the progression of tumor growth and metastatic spread. Tumor specific proteins produced by melanoma cells in vivo and in vitro are being studied, in order to determine the mechanism of their formation, to examine the impact of their expression on tumor growth, and to study the feasibility of utilizing their specificity for the immunoassay and immunotherapy of malignant melanoma. The control mechanisms involved in the regulation of pigment production in normal and in transformed melanocytes are also being characterized. The results indicate that various murine melanomas (of spontaneous, ultraviolet light induced, and chemically induced origin) share common cell surface antigens which are capable of eliciting a tumor rejection response; these antigens have a specificity restricted to melanoma cells. One melanoma tumor specific antigen has been purified to homogeneity and partially characterized; it is a glycoprotein of 65 kilodaltons, which has significant biochemical and immunological homology to serum albumin. This antigen has been shown to be effective in the immunization of mice against subsequent challenge with melanoma in transplantation assays, demonstrating the potential immunological significance of tumor specific antigen production to the progress of tumor growth. We have also produced monoclonal antibodies specific for the melanocyte specific enzyme, tyrosinase; these reagents cross-react with human tyrosinase and promise to be extremely useful for the identification of normal and transformed melanocytes in vivo and in vitro. They should further prove valuable for the study of cellular control mechanisms functional in the response of melanocytes to varying environmental stimuli which affect pigmentation, such as ultraviolet light and melanocyte stimulating hormone.

GPO 914-918

Other Professional Personnel:

Francesco Blasi	Director	Intl. Inst. Gen. Biophys., Milan
Miles Chedekal	Assoc. Professor	Johns Hopkins Univ., Baltimore
Umberto Ferrini	Professor	Regina Elena Cancer Inst., Rome
Douglas M. Gersten	Assoc. Professor	Georgetown Univ., Washington D.C.
John Marchalonis	Professor	Medical Univ. of SC., Charleston
Jesse Nicholson	Professor	Howard Univ., Washington D.C.
John Pawelek	Asst. Professor	Yale University, New Haven
Yasushi Tomita	Visiting Scientist	Tohoku Univ. Sch. Med., Sendai

Project Description

Objectives:

Our studies on the immune response(s) to malignant melanoma are directed towards the characterization of: (1) the nature of the host immune response elicited, (2) the effect of this response on the progression of primary tumor growth, and (3) the effect on the neoplasms's subsequent dissemination to metastatic sites. These studies are further targeted at (4) the isolation and characterization of the melanoma tumor specific transplantation antigens (TSTA) involved in these interactions. Our studies of tumor specific antigens (TSA) produced by transformed melanocytes are directed towards the identification and characterization of proteins abnormally expressed by transformed melanocytes, throughout their development in vivo, and the mechanism(s) involved in that altered expression. Lastly, studies on melanogenesis are directed towards the elucidation of the control mechanisms involved in regulating melanogenesis in normal and transformed melanocytes.

Methods Employed:

Murine melanoma cell lines being tested include parental B16 lines, several B16 sublines which differ with respect to their metastatic potential, the S91, and the recently derived JB/RH, JB/MS, and K1735 lines. Specificity controls include sarcoma, mammary adenocarcinoma, leukemia, neuroblastoma, and fibrosarcoma tumor cell lines. Tumor rejection studies are performed utilizing as immunogens: (1) irradiated tumor cells grown in vivo or in vitro with various schedules of inoculations, (2) viable tumor cells subcutaneously implanted in footpads, and removed by amputation of the primary tumor after several weeks. Immunized and naive (or sham-immunized) mice are then challenged with viable tumor cells, either subcutaneously (to determine effects on primary tumor growth), or intravenously (to characterize patterns of metastatic spread). The effect of host immune responses are monitored at routine intervals by palpation and measurement of the size of the growing primary tumor, or by counting of metastatic nodules in distant organs. Purification of the TSTA has thus far employed solubilization with aqueous butanol, salt fractionation and Sephacryl gel filtration chromatography. Purification and recovery of TSTA are monitored by in vivo tumor rejection studies. Final purification of the antigens is underway and will employ preparative PAGE and high performance liquid chromatography (HPLC), under nondenaturing conditions in order to maintain biologic activity. For purification of TSA, melanoma tissues

growing in vivo are excised and homogenized, and subcellular fractions are isolated and purified by means of an extensive series of differential centrifugations. Following gel filtration and preparative PAGE, mg quantities of the proteins under study have been isolated, allowing their characterization with regard to amino acid content, isoelectric point, molecular weight, prosthetic group content, amino terminal sequences, and peptide maps. In addition, polyclonal antibodies to these purified proteins have been produced in both rabbits and goats, and monoclonal antibody producing hybridomas from sensitized rat splenocytes have been cloned that are specific to the antigen.

The characterization of melanogenesis employs histochemical, biochemical, immunological, spectrophotometric, and radioactive assay methodologies. Melanocytes to be studied are from in vivo and in vitro sources; cells are fractionated into subcellular components by means of density gradient and differential centrifugations. After solubilization of melanosomes with nonionic detergents, the enzymes are further purified by gel filtration, preparative PAGE, and HPLC. Enzymes are then assayed with the appropriate substrates and controls under varying environmental conditions; the production of melanin and other reaction products can be followed spectrophotometrically, by liquid scintillation counting of newly formed radioactive precursors, by amino acid analysis, or by HPLC. Synthesis and processing of enzymes are analyzed using metabolic labeling of cells and immunoprecipitation by specific antibody probes.

Major Findings:

IMMUNE RESPONSE TO MALIGNANT MELANOMA

Our studies (Hearing and Law) have shown that the JB/RH (chemically induced), the K1735 (ultraviolet light induced), and the B16 (spontaneous), murine melanomas produce TSTA which not only display autologous tumor rejection activity, but share determinants with the heterologous melanoma lines as well. Our specificity studies show that this activity is restricted to the melanoma cell lines tested, and there has been no cross-reaction with any of the non-melanoma tumor lines tested to date, with the exception of neuroblastoma cells (which have an identical embryologic derivation). Interestingly, the S91 (spontaneous) pigmented melanoma, although highly immunogenic, does not appear to share cell surface TSTA with the other melanoma lines examined. Extraction of melanoma cells with aqueous butanol results in the quantitative extraction of TSTA, and has proven to provide an excellent initial solubilization and purification step. The TSTA has now been further purified and has an apparent molecular weight of 50 to 80 kilodaltons; final purification of this molecule to homogeneity is in progress. The cross-reacting TSTA found on the B16, the JB/RH and the K1735 melanomas are extremely interesting since these tumors were generated by radically different mechanisms, yet the TSTA do not display the typical unique specificity usually associated with TSTA from chemically induced and ultraviolet light induced neoplasms.

TUMOR SPECIFIC ANTIGENS OF MALIGNANT MELANOMA

Membrane proteins from transformed melanocytes vary in structure from those of normal melanocytes, and even from other types of transformed cells (Hearing, Gersten and Marchalonis). It has been found that tumor-specific proteins released from melanoma cells can be found in the serologic fluids of tumor bearing patients

and mice, and that large quantities of these proteins are shed from melanoma cells in vitro. It has been recently shown that one of these shed proteins has structural homology to the albumins, and may represent a normally occurring gene which is abnormally expressed in neoplastic tissues. Most recently, we have found that various subpopulations of melanoma cells growing in vivo after sequential transplantation become predominant, and that these differ in the structure of antigen expressed on the cell surface. Thus, TSA isolated from sequential preparations of melanoma tumors differed significantly in their amino terminal sequences, and this variability in antigen expression may be responsible for the diversity of subpopulations of melanoma cells that can be detected immunologically within a single tumor. In addition, the ability of this TSA to function as a TSTA has now been demonstrated (Hearing, Gersten, Galetto and Law). Thus, there are implications that these tumor-specific proteins may have a critical immunologic importance to the survival of the tumor in the host.

We have also studied the importance of the cell surface membrane expression of plasminogen activator on tumor invasion and metastasis; this enzyme has been postulated to be critical to the process of the tumor cell's penetration of the vascular endothelium during metastasis. We (Hearing, Blasi, Appella and Law) have been able to demonstrate that alteration of plasminogen activator activity on the melanoma cell surface has dramatic consequences on the subsequent metastatic behavior of these cells. Preincubation of B16 cells with specific antibodies which inhibit the enzymatic function of plasminogen activator significantly reduced the metastatic potential of those cells, while other, non-inhibitory, antibodies specific for plasminogen activator can actually enhance metastatic potential, perhaps by optimizing the binding of the cells to the substrata. Although the mechanisms involved have not yet been elucidated, these studies have shown that plasminogen activator plays an important role in the complex process of tumor metastasis.

REGULATION OF MELANOGENESIS

Since only one enzyme (tyrosinase) is essential for melanin biosynthesis, yet rates of pigmentation are influenced at many levels, including hormonal (melanocyte stimulating hormone) and environmental (ultraviolet light), the melanocyte provides a unique system for the study of cellular control mechanisms. It has always been questioned how tyrosinase, which is present in an active configuration in the endoplasmic reticulum and Golgi apparatus, is inhibited, but subsequently activated once in situ in the melanosome. Our studies (Hearing and Nicholson) have shown that ℓ-3,4-dihydroxyphenylalanine (ℓ-DOPA) is the natural activator of the enzyme in vivo; we have also shown that tyrosinase is extremely specific in its requirement for ℓ-DOPA as a cofactor, which apparently acts as an allosteric regulator. Our studies have also provided evidence that the synthesis of pigment may be further controlled by an as yet uncharacterized enzyme-associated factor(s) (Hearing, Chedekal and Pawelek). The latter may prove to be critical to the overall rate of pigment production in mammals, or the lack of melanogenesis, such as is evident in pigmentary disorders such as albinism and vitiligo. Our study has further resulted in the production of tyrosinase-specific monoclonal antibodies which should be invaluable for the characterization of post-translational enzyme processing in melanocytes (Hearing and Tomita). These probes also promise to be useful for the specific and sensitive identification of melanocytes in vivo and in vitro (in both normal and transformed populations).

Since these antibodies cross-react with tyrosinase in human melanocytes, they should also prove to be important for the study of normal and transformed melanocytes in human tissues. Studies are currently underway to characterize the response of melanocytes to such agents as melanocyte stimulating hormone, ultraviolet light and Vitamin D3, utilizing these tyrosinase specific monoclonal antibodies. Collaborative studies are also underway to determine the primary structure of tyrosinase, at least sufficiently to enable a specific nucleic acid probe to be constructed (Hearing, Ferrini and Appella); such a probe would be essential to molecular studies on the activation mechanisms for tyrosinase activity, and the eventual cloning of the gene for this important enzyme.

Significance to Biomedical Research and the Program of the Institute:

These studies are ultimately aimed at the evaluation of the factors involved in the synthesis and expression of tumor antigens on the surface of the neoplastic cell, and how these antigens might result in successful immunization of the host against the malignant cell. It should prove even more interesting to determine how these responses can be stimulated or abrogated, and how the immunologic defenses of the host are subverted under 'normal' conditions of tumor growth. Such information should prove to be extremely valuable to the understanding of the progress of tumor growth and metastasis in humans, where such experimental manipulations are not feasible.

Proposed Course of Research:

The studies as outlined above are currently in progress.

Publications:

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SUMMARY REPORT

LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY

October 1, 1984 to September 30, 1985

The Laboratory of Tumor Immunology and Biology carries out a range of laboratory investigations in tumor immunology, tumor cell biology, and molecular biology. Areas of particular emphasis include: (a) biochemical mechanisms of oncogene expression and cell growth; (b) the identification and characterization of cellular genes associated with the development of murine and human mammary neoplasia; and (c) the development and utilization of monoclonal antibodies directed against tumor associated antigens, with emphasis being placed on the study of human mammary and colon carcinomas.

The Laboratory of Tumor Immunology and Biology was established in 1982. It is composed of six Sections: The Cellular and Molecular Physiology Section (Dr. Herbert Cooper, Chief); The Biochemistry of Oncogenes Section (Dr. Robert Bassin, Chief); The Oncogenetics Section (Dr. Robert Callahan, Chief); The Experimental Oncology Section (Dr. Jeffrey Schlom, Chief); The Cellular Biochemistry Section (Dr. Cho-Chung, Chief); The Cell Cycle Regulation Section (Dr. William Kidwell, Chief).

CELLULAR AND MOLECULAR PHYSIOLOGY SECTION (Dr. Herbert Cooper, Chief)

The overall goal of projects currently in progress in this Section, and of those completed during the past year, is the elucidation of biochemical events involved in regulation of cell growth whose derangement as a consequence of oncogene expression may cause or contribute to neoplastic cell transformation. Three biochemical areas have been emphasized:

1. Alterations in the synthesis of specific cellular proteins as a consequence of expression of retroviral oncogenes in murine (NIH/3T3) cells.

2. Derangements in synthesis and organization of cytoskeletal proteins in human neoplasms and their mode of contribution to the neoplastic state.

3. Phosphorylation events related to the response of human leukemic cells to tumor-promoting phorbol esters.

Studies in area 3 have been brought to completion during the past year. Areas 1 and 2, because of interesting results of initial studies, have become the major areas of investigation currently being pursued in this Section.

In Project 1 we compare 2-dimensional electropherograms of newly synthesized proteins from lines of NIH/3T3 cells transformed by a variety of retroviral oncogenes, from flat cellular revertant lines, and from a line (433.3) which expresses the v-ras oncogene in response to corticosteriods. We detected 7 proteins whose synthesis was strongly suppressed in cell lines transformed by each of the six retroviral oncogenes we studied, and whose production was fully or partially restored in two cellular revertant lines. Suppression of two of these proteins was also correlated with the initial appearance of morphological alteration during steroid-induced oncogene expression in 433.3 cells. These proteins (p37/4.78 and p41/4.75) were identified as tropomyosins, a group of at least 5 cytoskeletal proteins. Transformation by the papova viruses, SV-40 and polyoma, caused no suppression of synthesis of these tropomyosins. This indicates that suppression of tropomyosin synthesis is not a nonspecific response by cells to being forced to grow with the transformed phenotype, but is specifically associated with oncogenesis by diverse retroviral oncogenes. The results are consistent with the hypothesis that the different biochemical processes initiated by expression of structurally diverse retroviral oncogenes may converge on a limited number of common targets, one of which is the mechanism which regulates the synthesis of tropomyosins.

In Project 2, the synthesis and content of tropomyosins and other cytoskeletal proteins in various human tumor cell lines and tissues is being investigated in order to determine whether synthesis of these proteins is suppressed in human neoplasms as it is in NIH/3T3 cells transformed by oncogenic viruses. Tropomyosins synthesized by various tissue types will be identified by 2-dimensional polyacrylamide gel electrophoresis on the basis of molecular weight, isoelectric point, cytoskeletal localization, absence of tryptophan and immunological reactivity. Expression of tropomyosins in tumor cells of these types will then be evaluated. Synthesis of other cytoskeletal proteins will be evaluated in the same preparations. The following systems will be studied: lymphoid cells, myeloid cells, colon carcinoma, bladder carcinoma, and mammary carcinoma.

In Project 3, which has been brought to completion and will not be continued, phosphorylation of proteins during response of cells to treatment with phorbol esters (PMA) was studied. In HL-60 promyelocytic leukemia cells, growth arrest and differentiation after PMA exposure are associated with rapid phosphorylation-dephosphorylation of proteins pp17 and pp27. Cell-free studies suggest that this may involve the activation and cooperation of two classes of protein kinase, calcium-phopholipid-dependent kinase and cAMP-dependent kinase. Significantly, enhanced phosphorylation of pp17 and pp27 was found only in cell lines where PMA caused growth arrest and differentiation. The effect was minimal in cells where PMA was mitogenic. PMA also induces aggregation of platelets during which increased protein phosphorylation occurs. Elevated phosphorylation of class-I HLA molecules was documented, together with evidence suggesting association of HLA in a complex with myosin and actin and implicating modification of HLA as a component of platelet activation.

BIOCHEMISTRY OF ONCOGENES SECTION (Dr. Robert Bassin, Chief)

The Biochemistry of Oncogenes Section studies the mechanisms of cell transformation and tumor formation by retroviral oncogenes. Two general approaches are employed: analysis of flat revertant cells resistant to transformation by certain groups of oncogenes; and biochemical studies, in which the role of cell membrane components in growth and transformation are examined.

A new flat revertant cell line, isolated after treatment of Ki-ras-transformed NIH/3T3 cells with the mutagen MNNG and selection in 1 mM ouabain, has been studied. Like the C-11 and F-2 cell lines previously reported, the new line, Clone 22, contains elevated amounts of the ras gene product p21 and rescuable Ki-MuSV, but exhibits many of the growth properties of normal NIH/3T3 cells. Clone-22 cells are resistant to transformation when challenged with viruses containing the oncogene ras.

As a preliminary step to cloning and identifying the gene or genes responsible for the revertant phenotype, high molecular weight DNA has been isolated from Clone 22 cells. Following transfection of Clone 22 DNA onto recipient ras-transformed cells, a small number of colonies consisting of flat, apparently nontransformed cells can be seen. A preliminary restriction map has been made by cleaving Clone 22 DNA with each of 8 different restriction endonucleases and noting the frequency of appearance of flat colonies following transfection of each DNA digest. Preparations to clone the gene responsible for the nontransformed phenotype are in progress.

Revertant cells were originally selected on the basis of their resistance to the toxic effects of ouabain as compared to ras-transformed cells. Earlier transport studies had indicated that Na^+, K^+ ATPase, the specific enzyme target of ouabain, was not different in normal, transformed and revertant cell lines. We have now examined the activity of Na^+, K^+ ATPase in the same cell lines directly in an in vitro ATPase assay, and the results support our original conclusion. Studies examining other ion transport pathways in normal and transformed cells are in progress.

Studies of proteins synthesized by normal, transformed and revertant cells by 2-dimensional gel electrophoresis have indicated that surprisingly few changes are invariably associated with transformation by retroviral oncogenes. Two such proteins, p 38 and p41, have now been identified as tropomyosins, a group of at least 5 proteins which are associated with the cytoskeleton. Changes in tropomyosin synthesis have been associated with transformation by all 6 of the retroviral oncogenes tested to date but not with transformation by the papovaviruses SV_{40} and polyoma.

The mechanism of transformation by retroviral oncogenes is also being studied through a biochemical analysis of transmembrane signal transmission systems which are known to be involved in cell growth regulation. It was previously shown that the activation of protein kinase C, a cellular receptor for phorbol ester tumor promoters, can be correlated with cell growth and transformation. Protein kinase C activity is predominantly membrane-associated in rapidly growing,

low population density or phorbol ester-treated cells, but becomes more associated with the cytosol in growth arrested, high population density cells.

Interleukin 2 causes a rapid, transient redistribution of protein kinase C from cytoplasm to nucleus in CT6 cells - the same effect as was noted following treatment with phorbol ester, although in the latter case the effect was of a longer duration. Interleukin 3 has a similar effect on FDC-P1 cells. These results are evidence that the distribution and activity of protein kinase C can be modulated by growth factors. Studies with simian and Kirsten sarcoma viruses indicate that cells transformed by these retroviruses also cause a redistribution of protein kinase C so that a larger proportion of the enzyme becomes associated with the cell membrane.

ONCOGENETICS SECTION (Dr. Robert Callahan, Chief)

The Oncogenetics Section conducts research to identify and molecularly clone tumor specific and viral genetic elements from human and other mammalian species. The characterization of these recombinant clones includes a description of their organization in cellular DNA, their chromosomal location and their biological activity in normal and neoplastic tissue. The ultimate aim is to develop an understanding of the genetic changes that occur and their consequences in the etiology of human neoplasia. During the past three years, the Oncogenetics Section has focused its efforts in three areas: (1) characterization of the genetically transmitted (endogenous) mouse mammary tumor virus (MMTV) genomes in inbred and feral strains of mice, and their potential role in tumorigenesis; (2) identification of the cellular genes at risk in mammary tumors induced by infectious MMTV; and (3) characterization of MMTV related sequences in human cellular DNA.

Studies of endogenous MMTV genomes in the BALB/c inbred mouse strain have demonstrated the expression of a novel 1.6 kbp MMTV mRNA in normal lactating mammary gland and pristane induced plasma cell tumors. This species of MMTV mRNA is composed primarily of sequences from the long terminal repeat (LTR) element of the viral genome. The MMTV LTR contains an open reading frame which encodes a 26-30 k Dalton protein. The function of this protein in viral replication and the consequences of its expression in plasma cell tumors is unknown. To help further define at the genetic level the role of the BALB/c MMTV proviral genomes (Unit I, II and III) in neoplasia, we have identified their chromosomal location. Results of this analysis show that Unit I, II and III are located on respectively chromosomes 16, 6 and 12.

The activation of two cellular genetic loci (designated int-1 and int-2) by the insertion of an MMTV genome is associated with mammary tumor development in high incidence inbred strains of mice. We have expanded the scope of these studies to feral species of mice. Recently, we have identified maternal lineage groups of M. cervicolor popaeus which have a high incidence of mammary tumors and contain an MMTV related virus (MC-MTV). Examination of tumor DNA demonstrated an association between MMTV induced rearrangement or activation of the int-1 and int-2 loci and mammary tumor development in 50% of the tumor bearing mice. These results extend the previous findings in inbred mouse strains to a

distantly related, and reproductively separate, species of mice. In addition, these results show that activation of these cellular genes in mammary tumors does not appear to be linked to highly infectious laboratory strains of MMTV. In another study, the feral M. musculus musculus (Czech II) strain of mice were found to lack endogenous MMTV genomes in their germline, but are infected with an exogenous MMTV related virus. Examination of mammary tumors from these mice has led to the identification of a new common integration locus (int-3) for MMTV in tumor DNA. The int-3 locus is unrelated to the int-1 and int-2 loci as well as to other cellular oncogenes. It is located on mouse chromosome 17.

We have previously identified and obtained recombinant clones of MMTV related sequences in human cellular DNA. Recently, we have been able to show that the MMTV gag-pol related sequences are located within a novel class of human endogenous retroviral genomes. The human proviral genome contains a mosaic of sequences characteristic of different retroviral genera. There are approximately 50 to 100 copies of these proviral genomes in human DNA. The human proviral LTR-like element has been disproportionately amplified (1000 copies) in human cellular DNA. This class of endogenous retroviral genomes has been intimately associated with several primate species during the course of evolution.

The current focus of our efforts is: (1) the molecular and biological characterization of the int-3 locus; (2) the identification of new int loci in MMTV induced mammary tumors of feral mice; (3) the completion of the molecular and biological characterization of the human endogenous retroviral genomes; and (4) the determination of the contribution of this class of human retroviral genomes to the development of neoplasia. In the future, we anticipate expanding the scope of our program to include the identification and characterization of the cellular genes at risk in human breast and colon carcinomas and dimethylbenzanthracene induced mammary tumors of Czech II mice.

EXPERIMENTAL ONCOLOGY SECTION (Dr. Jeffrey Schlom, Chief)

The major research goals of the Experimental Oncology Section (EOS) include the study of the cell biology and tumor immunology of human carcinomas with emphasis being placed on the study of human breast and colon cancer. It is anticipated that the studies outlined here involving the identification and/or characterization of tumor associated antigens, growth and tumor factors and their receptors, oncogene products and epithelial differentiation antigens within carcinoma cells may lead to: (i) a better understanding of the biologically relevant phenotypes that exist among carcinoma cell populations, (ii) a clearer definition of the multistep processes involved in carcinoma pathogenesis, and (iii) the development of reagents and assays that may eventually prove useful in several areas of the management of specific human carcinomas.

The studies being conducted in the EOS have been divided into several projects as summarized below.

Monoclonal Antibodies Define Carcinoma Associated and Epithelial Differentiation Antigens. These studies involve the generation and utilization of monoclonal antibodies (MAbs) to identify and characterize human carcinoma associated antigens and differentiation antigens of mammary and colonic epithelium. These MAbs are being used to better understand the cell biology and pathogenesis of human carcinomas, and to provide potential reagents and assays for use in several aspects of the management of human carcinomas. These include: detection of occult tumor cells; further defining the degree of differentiation of "normal", dysplastic, and carcinoma cell populations; development of serum antigen assays; and radiolocalization of primary and metastatic carcinoma lesions in situ (and potentially therapy) using radiolabeled monoclonal immunoglobulins and fragments. These studies are divided into four areas of investigation: (I) The generation and characterization of an MAb (B72.3) that defines a novel tumor associated antigen (TAG-72). This antigen is a high molecular weight (>10^6d) glycoprotein that has been shown to be selectively expressed in the majority of human colon, mammary, and ovarian carcinomas versus benign counterparts and normal adult tissues. MAb B72.3 is currently being evaluated for use in the detection of occult tumor cells, serum antigen assays, and detection of carcinoma lesions in situ using radiolabeled immunogloublin; (II) The development and characterization of MAbs to a repertoire of epitopes on carcinoembryonic antigen (CEA) which are differentially expressed among carcinoma cell populations. These MAbs have been divided into five groups based on their differential reactivity to various carcinoma cell populations. Six of these MAbs have been extensively characterized and have shown a great deal of selective reactivity for colon carcinoma cells versus adult normal tissues, including those rich in CEA "cross-reactive" antigens; (III) The generation and characterization of MAbs to proteins associated with metastatic cell populations; MAbs to the 68K laminin receptor have defined the expression of this molecule in both malignant and benign mammary and colon epithium; and (IV) The definition and characterization of breast and colon differentiation antigens. MAb DF3 has been used to distinguish the 290K antigen distribution in benign versus malignant mammary epithelium and has been shown to be an independent marker of mammary tumor cell differentiation. MAb CDA has been used to distinguish and physically separate poorly differentiated from well differentiated human colon carcinoma cell populations. These MAbs are thus currently being used, or will be used, in studies to better define the "premalignant" phenotype, to differentiate presently indistinguishable carcinoma cell populations that may differ in biologic properties, and to provide potential clinically useful reagents.

Analyses of ras p21 Oncogene and Proto-Oncogene Expression in Human Carcinomas. Monoclonal antibodies (MAbs) of predefined specificity have been generated by utilizing a synthetic peptide reflecting amino acid positions 10-17 of the Hu-ras^{T24} gene product as immunogen. When paraffin-embedded Formalin-fixed tissue sections and the avidin-biotin complex immunoperoxidase methods were used, the RAP (RA, ras; P, peptide) MAbs clearly defined enhanced ras p21 expression in the majority of human colon and mammary carcinomas. The majority of all abnormal ducts and lobules from fibroadenoma and fibrocystic disease patients were negative, as well as normal mammary and colonic epithelia examined. We have used the RAP MAbs to define ras p21 protein expression in a spectrum of colonic disease states. Immunohistochemical analyses of individual cells

within tissue sections reveal differences in ras p21 expression in colon carcinomas compared with normal colonic epithelium, benign colon tumors and inflammatory or dysplastic colon lesions. Our data suggest that ras p21 expression is correlated with depth of carcinoma within the bowel wall, and is probably a relatively late event in colon carcinogenesis.

We have recently developed a quantitative liquid competition RIA for ras p21 using MAb Y13259. By the use of a standard of pure recombinant ras p21, we are now able to detect p21 at fM levels, and to determine the number of molecules of p21 per cell. The concomitant use of this quantitative RIA and immunohistochemical analyses of normal, dysplastic and inflammatory disease states, as well as carcinomas, should lead to a better understanding of the role of the ras gene in the genesis of these lesions.

Epidermal Growth Factor (EGF) and Transforming Growth Factors (TGF) in Rodent Mammary Tumor and Retrovirus Transformed Cells. A variety of viral and chemically transformed cells and spontaneously arising tumor cells produce a family of growth regulators collectively known as transforming growth factors (TGFs). TGFs are able to confer upon normal, nontransformed cells several properties associated with the transformed phenotype and may be involved in the autocrine growth of these cells. Some of these TGFs, namely alpha-TGF, are structurally related to epidermal growth factor (EGF). EGF is required for the growth and survival of normal rodent mammary epithelial cells in a serum-free, hormone-defined medium. However, in contrast, rat mammary tumor cells obtained from chemically-induced rat mammary adenocarcinomas exhibit a diminished response to EGF. This reduced responsiveness to EGF is due to the production of an alpha-TGF-like growth factor, mammary tumor factor (MTF), by these tumor cells. MTF is a heat labile protein whose activity is destroyed by reduction. MTF exists as two species with molecular weights of 68,000 and 6,000 and a pI of 5.2. MTF is mitogenic for normal mammary epithelial cells but not for the tumor cells from which it was derived. Since chemically-induced rat mammary tumors have either an elevated expression or an alteration in structure of the oncogene protein, $p21^{ras}$, we examined the potential role of TGFs in the transformation of NIH/3T3 cells produced by Kirsten murine sarcoma virus (v-Ki-ras onc gene) and in two cellular revertants (C11 and F2) derived from v-Ki-ras transformed 3T3 cells (DT). The revertant cell lines possess elevated levels of $p21^{ras}$ like DT cells yet fail to grow in soft agar or are tumorigenic in nude mice. However, like DT cells, the revertants lack detectable EGF receptors and produce TGFs. Unlike DT cells, the revertants fail to grow in soft agar even in the presence of exogenously supplied TGF. These studies suggest that the lesion(s) in the revertants are distal to the elevated expression of $p21^{ras}$ and production of TGFs and that the elevated production of TGFs is necessary, but may not be entirely sufficient for maintaining the transformed phenotype in ras transformed cells.

Isolation and Characterization of Transforming Growth Factors from Human Mammary Tumors and Human Milk. The presence and role of transforming growth factors (TGFs) in human mammary carcinoma cells is being defined. Alpha-TGFs resemble epidermal growth factor (EGF) in that they are able to compete with EGF mouse and human EGF but fail to cross-react with antibodies generated against these two species of EGF. Alpha-TGFs can be recovered from the acid-treated,

concentrated conditioned medium (CM) of a human mammary carcinoma cell line, MCF-7, and ten individual clones derived from this cell line. The level of TGF activity associated with the CM varied amongst the different MCF-7 clones and showed no correlation with either the number of EGF receptors expressed on these cells or with the intrinsic ability of these clones to grow in soft agar as colonies. The TGF in the CM is an acid heat stable peptide whose activity is destroyed by reduction. It has a molecular weight of approximately 6000 and a pI of 4.0 TGF activity can also be detected in the crude, acid-ethanol extracts prepared from MCF-7 cells propagated as tumors in nude mice, in the acid-ethanol extracts obtained from two transplantable human mammary adenocarcinomas, Clouser I and II, and in 3 out of 4 primary human breast carcinomas samples. Relatively high levels of TGF activity can be found in crude, delipidated, decaseinated human milk obtained from at least 30 individual donors. The levels of TGF in the milk samples vary from individual donors and are generally highest in colostrum. Following isoelectric focusing (IEF), three distinct TGF species can be detected in the human breast tumor samples with identical species found in human milk. The TGF with a pI of 3.8 - 4.0, mammary-derived growth factor-II (MDGF-II), has been purified approximately 10,000-fold. This alpha-TGF species is virtually identical to the species found in the CM from MCF-7 cells. This acidic alpha-TGF is biologically and physiochemically distinct from the major species of human EGF in milk.

Factors Regulating the Expression of Tumor Antigens in Human Carcinoma Cell Populations: Implications for Understanding Antigenic Heterogeneity and Augmenting Monoclonal Antibody Binding. Within human breast and colon carcinoma lesions, as well as established human tumor cell lines, there exists extensive heterogeneity in the expression of defined tumor antigens as recognized by the binding of monoclonal antibodies (MAbs). This heterogeneity of tumor antigen expression can be found within defined areas of the tumor and within subcellular compartments of a single tumor cell. These studies have shown that human tumor cells have the ability to intrinsically regulate the expression of the tumor antigens. Factors such as 1) cell cycle kinetics, 2) long-term growth in vitro, 3) clonal variability, and 4) growth of tumor cells in three-dimensional structures all alter the antigenic phenotype of human tumor cells. The apparent ability of human tumor cells to intrinsically modulate their tumor antigen expression provides insight into the factors contributing to the extensive antigenic heterogeneity observed in these cell populations. Studies were conducted to identify those biological response modifiers that can mediate an increase in tumor antigen expression and thus increase the cell surface binding of monoclonal antibodies directed against those antigens. Recombinant human leukocyte interferon (IFN-alpha A) was found to increase the binding of specific MAbs to the surface of human breast and colon carcinoma cells in a dose-dependent manner. Utilizing 10 to 100 units IFN-alpha A, tumor antigen expression can be dramatically increased independent of any change in tumor cell proliferation. Cell sorter analysis revealed that the IFN-alpha A mediated increase in tumor antigen expression is a result of an increased amount of antigen per cell as well as the recruitment of previously antigen negative cells to antigen positive. These studies may thus lead to: (i) a better definition of the transformed phenotype and (ii) the development of new strategies to enhance the in vivo binding of monoclonal antibodies to tumor antigens.

The Use of Monoclonal Antibodies and Immunohistochemistry and Immunocytochemistry to Detect and Phenotype Carcinoma Cell Populations. A monoclonal antibody (MAb), designated B72.3, has been generated using membrane enriched fractions of a metastatic human breast carcinoma as the immunogen. Previous studies have demonstrated that the reactive antigen, a novel high molecular weight glycoprotein complex, can be detected in Formalin-fixed paraffin-embedded tissue sections of human breast and colon carcinomas, and not in a variety of normal adult human tissues. We have now determined that MAb B72.3 may be used as an adjunct for diagnosis of adenocarcinoma in cytologic preparations of human effusions. Using the avidin-biotin complex method of immunoperoxidase staining and Formalin-fixed paraffin-embedded cell suspensions, MAb B72.3 detected adenocarcinoma cells in effusions from all of 21 patients with adenocarcinoma of the breast. No reactivity was demonstrated in any cell type in benign effusions from 24 patients without cancer, or 13 patients with prior or extant cancer in other body sites; moreover, B72.3 showed no reactivity to leukemic or lymphomatous effusions, or apparent mesothelial cells from malignant effusions. Monoclonal antibody B72.3 also detected adenocarcinoma cells in cytologic effusion specimens from 12/12 patients with adenocarcinoma of the lung and 16/16 patients with adenocarcinoma of the ovary. Thus, these data suggest that the immunocytological application of MAb B72.3 should now be considered as an adjunct in the discrimination of adenocarcinoma cells from reactive mesothelial cells in the cytologic diagnosis of malignant effusions and may be applicable in the detection of occult tumor cells in needle aspirates as well as other biologic fluids.

Characterization and Purification of a Tumor Associated Antigen (TAG-72) and Establishment of Radioimmunoassays for TAG-72. A competitive radioimmunoassay (RIA) for a tumor associated antigen (termed TAG-72) identified by monoclonal antibody B72.3 has been established. The RIA was used to examine sera from patients with colorectal carcinomas, other malignancies and normal sera. A mean of 2.2 units/ml of TAG-72 was found in the normal sera. When a cut-off level of 3 standard deviations above the mean level of TAG-72 found in normals is used, no patient with inflammatory disease or other benign colon diseases exhibited abnormal levels of TAG-72. Thirty-five percent of sera from advanced colon cancer patients and patients with other carcinomas were positive for TAG-72. No increase in TAG-72 reactivity was seen in normal sera or that from patients with melanomas or sarcomas. Comparison of the TAG-72 levels in sera with antigens recognized by the monoclonal antibodies currently used to screen sera of carcinoma patients clearly demonstrated that TAG-72 is different from the other antigens, and that TAG-72 can be found in some sera where no antigen is detected by these other MAb RIAs.

TAG-72 has been partially purified from extracts of a human colon carcinoma xenograft in athymic mice using molecular sieving and antibody affinity chromatography. It has an apparent molecular weight of $\geq 10^6$ daltons, and has been characterized as a mucin.

The Use of Monoclonal Antibodies for Tumor Localization and Therapy: Experimental Models. Monoclonal antibodies B6.2 and B72.3 bind to human breast and colon tumor associated antigens. IgG from both these monoclonal antibodies was purified and F(ab')2 and Fab' fragments were prepared from the B6.2 IgG. The B72.3 IgG

and B6.2 IgG and fragments were radiolabeled with I-125 and I-131 without loss of their immunoreactivity. The radiolabeled antibodies and fragments were injected into athymic mice bearing antigen positive human breast or colon tumors or an antigen-negative melanoma as a negative control. The B6.2 IgG and fragments localized specifically in the breast tumor xenografts with the IgG giving maximal activity in the tumor. The F(ab')2 fragment gave the best tumor-to-normal tissue ratios (15-20:1 for liver and spleen) due to its rapid clearance from the blood stream. The Fab' fragment cleared more rapidly than the F(ab')2 or the intact IgG with the majority of the radioactivity in the kidneys. When radiolabeled B72.3 IgG was injected into mice bearing colon carcinoma xenografts activity in the tumor rose for the first 2 days and remained constant over the 19 day period of study. Tumor-to-normal tissue ratios rose over this period of time with ratios of approximately 18:1, for liver, spleen and kidney, at 7 days. At 19 days approximately 40% of the radiolabeled B72.3 IgG was found in the tumor. Model systems that resemble the metastatic nature of human colon carcinomas are being developed to better determine the efficacy of radiolabeled monoclonal antibodies as potential agents for radioimmunodetection and radioimmunotherapy.

Clinical Trials for the Detection and Localization of Carcinoma Using Radiolabeled Monoclonal Antibodies. Accurate detection and anatomic localization of both primary and metastatic lesions remains one of the major problems in the management of most human carcinomas. We have recently initiated clinical trials at the NIH Clinical Center to detect and localize colorectal carcinoma lesions using radiolabeled MAb B72.3. Parameters that are being or will be systematically investigated concerning both the efficiency of MAb localization and the efficiency of gamma scanning of carcinoma lesions include: (a) effect of MAb dose and specific activity of radionuclide coupled MAb; (b) comparison of the use of intact IgG, $F(ab')_2$, and Fab'; (c) choice of radionuclide;(d) route of inoculation; (e) size, location, and other inherent properties of the tumor mass such as antigen content; (f) the presence of circulating antigen; (g) the presence and/or absence of human anti-murine Ig antibodies; (h) metabolism of MAb and fragments; (i) combinations of MAbs. Biopsy material removed at surgery (both tumor and normal tissues) from patients who have received radiolabeled MAb are being directly analyzed to define the "radiolocalization index" or potential "therapeutic index" (i.e., the ratio of the amount of MAb bound [via cpm] per gram of tumor tissue to that bound per gram of normal tissues). It is hoped that these studies will also aid in establishing a rational basis for the subsequent therapeutic use of a particular MAb, either coupled to toxins, via effector cell-mediated or complement-mediated mechanisms, or using MAbs radiolabeled with one of a variety of isotopes.

CELLULLAR BIOCHEMISTRY SECTION (Dr. Yoon Sang Cho-Chung, Chief)

The objective of this project is to understand the mechanism underlying the control of growth of hormone-dependent mammary tumors. In previous work it was shown that cAMP antagonizes the estrogen action and produces growth arrest of hormone-dependent rat mammary carcinomas induced by DMBA. In the regressing tumors induced by either hormone-withdrawal (ovariectomy) or DBcAMP treatment,

cAMP receptor (cAMP binding protein) level increased while estrogen receptor level decreased in a strictly inversed manner. It was proposed that antagonistic interaction between estrogen and cAMP may be involved in the growth control of hormone-dependent mammary tumors. In fact, in rat mammary tumors, the ratio of steroid receptor to cAMP receptor was found to better discriminate hormone-dependent from independent tumors than steroid receptor alone. These results were confirmed in a limited number of primary human breast carcinomas examined. Moreover, determination of cAMP receptor level appeared to be a prognostic value for human breast cancer.

In the regression of mammary tumors induced by either hormone-withdrawal or DBcAMP treatment, the nuclear translocation of cAMP receptor protein is an indispensable event. This finding led to the studies of the effect of cAMP on gene expression in mammary tumors. The *in vitro* translation studies showed a marked decrease of the two protein bands (35 and 22K M.W.) and an increase in one protein band (20.5K M.W.) in the translated proteins from mRNAs obtained from regressing tumors of ovariectomy or DBcAMP treated rats. Changes in these translation products were reversed when tumor growth was stimulated by estrogen or cessation of DBcAMP treatment. The 22K M.W. protein was immunopercipitated by a rat monoclonal antibody directed against p21 *ras* transforming protein of Ha-MuSV. Immunoblotting experiments definitely identified the 22K M.W. protein to be *ras* p21. Thus, in hormone-dependent rat mammary tumors, c-*ras*^{H} proto-oncogene is expressed in growing but not in regressing tumors. The relationship between enhancement of c-*ras*^{H} expression and hormone-dependent growth of mammary tumors was examined in more than 200 human breast cancers. Immunoblotting experiments revealed a 10-fold elevation of p21 level above that of normal breast tissue in 70% of estrogen receptor positive breast carcinomas and in 40% of receptor negative tumors. In early breast cancers, importantly, the elevation of p21 was strictly restricted in the estrogen receptor positive tumors. The mechanism of hormone effect on p21 elevation in mammary tumors is currently being investigated.

Whether the growth regulatory effect of cAMP involves regulation of oncogene expression was investigated using clone 13-3B-4, a Ha-MuSV DNA transfectant of NIH-3T3 cells growing in a serum free defined medium. Treatment of cells with various cAMP analogs resulted in a marked decrease of p21 *ras* protein synthesis. The decrease in p21 levels in 13-3B-4 cells following cAMP treatment correlated with a change in the molecular species of cAMP receptor proteins, the regulatory subunits (R^{I}, R^{II}) of cAMP-dependent protein kinase type I and type II. In the cells treated with cAMP analogs, the concentration of R^{II} tripled while R^{I} content decreased to one-fifth of the untreated cell level, resulting in a ratio of R^{I}/R^{II} closely resembling that of untransformed 3T3 cells. These changes in the levels of p21 and cAMP receptor proteins in 13-3B-4 cells correlated with a change in morphology. Cells treated with cAMP analogs exhibited a morphology characteristic of untransformed fibroblasts, while the untreated cells retained a transformed phenotype. Thus, a role for cAMP and its recptor protein in the quantitative modulation of v-*ras*^{H} oncogene expression is demonstrated. The mechanism of this cAMP action at genomic level is currently being investigated.

CELL CYCLE REGULATION SECTION (Dr. William Kidwell, Chief)

Growth factors that stimulate the proliferation of normal and neoplastic mammary cells have been purified from rodent and human breast tumors and from human milk. The factors from the latter two sources have been purified to apparent homogeneity and are probably identical. This factor (MDGF1) exerts its biological effects via specific, high affinity, membrane receptors. However, evidence for a synergism with estrogen for proliferation has been obtained. The mammary cell's ability to respond to MDGF1 is dependent on the substratum on which the cells are grown. Stromal collagen and fibronectin potentiate the response while laminin and basement membrane collage inhibit it. These effects may be due to modulation of MDGF1 receptors. EGF and MDGF1 responsiveness of mammary cells are similarly affected by the culture substratum. EGF receptor number and affinities are the same on all four substrata when cells are grown in the absence of EGF. However, in the presence of a receptor down-regulating level of EGF, the cells regenerate membrane receptors more efficiently on stromal collagen and fibronectin than on type IV collagen or laminin. This difference in receptor number (2-3 fold) may explain the differing responsiveness to growth factors on the various substrata. It could be physiologically relevant since the epithelium is believed to penetrate the pre-existing basement membrane and contact stromal elements as it invades the surrounding tissue in response to a proliferatiave stimulus. Maintenance of a sufficient number of growth factor receptors could assure that the cells are competent to regenerate a new basement membrane since both EGF and MDGF1 greatly potentiate the cells ability to make new collagen via elevating collagen mRNA levels in cells.

A series of proline analogs have been analyzed for their effects on collagen synthesis inhibition in cultures of primary DMBA-induced rat mammary tumors and for their effects on mammary tumor growth in tumor bearing animals. Azetidine carboxylate, thioproline and cis-hydroxyproline were found to be potent, selective inhibitors of collagen synthesis, blocking amino acid incorporation into collagen by 7 to 27 fold more than incorporation into total tumor cell protein. In vivo all 3 of these compounds at doses of 50-200 mg/kg S.C., caused tumor growth arrest or regression. The conditions favoring proline analog sensitivity of mammary tumors have been assessed. A positive correlation exists between the ability of a tumor to synthesize basement membrane and its analog concentrations which affect tumor growth. Sensitivity is approximately proportional to the efficacy of the analog in blocking collagen synthesis in cultured tumor epithelium. The epithelium of normal mammary glands and mammary adenocarcinomas is dependent on proline for optimal growth, especially when cells are plated on stromal collagen substrata. Blocking basement membrane deposition and thereby favoring tumor cell contact with stroma may, therefore, promote proline analog uptake and tumor cell kill. In contrast to primary tumors, metastatic rat mammary tumor growth was not affected by proline analogs. Electron microscopy revealed that these tumors lacked a basement membrane. Using cDNAs against the collagen of basement membranes, type IV collagen, we have demonstrated a lack of collagen IV mRNA sequences in the metastatic tumors but the collagen message is present in collage IV producing, proline analog sensitive, primary tumors.

Poly(ADP-ribose) synthetase is a chromatin bound enzyme that adds chains of ADP-ribose in tandem to nuclear proteins. This enzyme is activated by DNA damaging agents such as gamma, x-ray and u.v. irradiation and by DNA alkylating agents. We have synthesized and tested 6 compounds which are inhibitors of the synthetase and found that the ability of 4 of 6 of them to block DNA repair is directly correlated with the compound's potency as a synthetase inhibitor. The compounds ranked in order of their ability to block DNA repair are 3-acetlaminobenzamide> 3-hydroxybenzamide= benzamide>>3-aminobenzamide. 3-nitrobenzamide, was found to be much more inhibitory for the repair of DNA chain breaks than was expected based on its potency as a poly(ADP-ribose) synthetase inhibitor. Plots of the reciprocal of the repair velocity vs inhibitor concentration normalized against its k_i for synthetase were made. These plots were biphasic indicating that the benzamides had effects on more than one cellular process. At least two processes other than DNA repair have been implicated as targets. These are RNA synthesis and glutamine synthetase. The latter enzyme was found to be an acceptor protein for mono-ADP-ribose. The enzyme catalyzing this reaction was also found to be inhibited by the benzamides, though their potency as inhibitors for this enzyme was much less than their potency as synthetase inhibitors.

Hormones and Growth Factors in Development of Mammary Glands and Tumorigenesis
(Dr. Barbara K. Vonderhaar and co-workers)

Studies have been designed to understand the role of hormones and growth factors in normal mammary gland development and differentiation. Studies include: 1) examination of the role of epidermal growth factor and mammary gland-derived growth factors in lobulo-alveolar development of the mouse mammary gland, 2) defining the roles of estrogen and progesterone in priming the mammary tissue prior to whole organ culture to determine their effects on induction of EGF receptors, mammary gland-derived growth factor receptors and the production of growth factors by the animals, 3) examine the hormonal conditions in vitro which induce production of autocrine growth factors by normal mammary tissue and breast cancer cell lines.

Studies have been undertaken to evaluate the nature of lactogenic hormone receptors and the factors (including other hormones) which affect binding of the hormone to this molecule. Studies include 1) purification of the receptor from human tissue and preparation and characterization of an antibody against it; 2) examininations of the nature of the subunits of the receptor, and 3) characterization of the nature of the interaction of Tamoxifen with membrane-bound receptors related to the lactogen receptor.

Gene Structure, Sequence and Regulation of the Expression of N-Acetylglucosaminde β1→4 Galactosyltransferase and its Modifier Protein - the Mammary α-Lactalbumin
(Dr. Pradman Qasba and co-workers)

Galactosyltransferases are the family of enzymes which transfer galactose from UDP-Gal to the nonreducing residues of oligosaccharides of various glycoconjugates as well as to monosaccharides resulting in a specific linkage of galactose to a specific acceptor molecule. Common to all of these galatocyltransferases is that they bind UDP-galactose. However, the specificity of each transferase lies in

generating a specific glycosidic linkage by transferring galactose to a specific acceptor molecule. The most common carbohydrate sequence, N-acetyllactosamine (Galβ1→4GlcNAc) is formed by N-acetylglycosaminide β1→4galactosyltransferase (Gal.Tra.) by transfer of galactose to free or bound N-acetylglucosamine generating β1→4 glycosidic linkage. The carbohydrate structure, Gal β1→4GlcNAc, or its repeating unit occurs on glycoproteins and glycolipids of various cell types and tumors and is recognized as a developmentally regulated antigenic determinant of human erythrocytes, and Type 2 blood group etc. In mammary gland during lactation the levels of this enzyme and its modifier protein α-lactalbumin (α-LA) increases. They are secreted in milk together with other glycosyltransferases.

Gal.Tra. activity is modified by α-LA-like molecules which alter the acceptor specificity of the enzyme. Mammary α-LA modifies the acceptor specificity of Gal.Tra. in a way which promotes the transfer of galactose to glucose to produce lactose and inhibits the transfer to N-acetylglucosamine. Several laboratories including ours have found in rat epididymal fluids and testis molecules similar to mammary gland α-LA but distinct in their modifier activity. Epididymal α-LA-like activity differs from mammary gland α-LA activity in that it transfers galactose to either glucose or myo-inositol with equal efficiency. These results have raised the possibility that α-LA-like proteins may interact with Gal.Tra. in transferring galactose to specific sugars either free or linked to membrane bound glycoproteins, resulting in a specific oligosaccharide sequence which may be recognized as a new antigenic determinant or specific differentiation antigens.

Mammary α-LA has been shown to be structurally related to lysozyme, an enzyme catalyzing the hydrolysis of β1→4 glycosidic linkage in polysaccharides. Based on the model building, the conformation of the main chain of α-LA, though for the most part similar to lysozyme, differs from it at the C-terminal portion. The C-terminal residues of α-LA are also essential for its interaction with Gal.Tra. It has been proposed that α-LA and lysozyme have arisen from a common ancesteral gene. The sequence of the entire mammary α-LA gene from our laboratory has shown that α-LA and lysozyme genes contain three introns at similar positions. The first three exons of the two genes have similar nucleotide sequences. But the two genes have diverged in the fourth exon. They show no statistically significant similarities and are markedly different. This fourth exon of α-LA codes for the C-terminal portion of the protein, which is conformationally different from the corresponding region of lysozyme and is essential for its interaction with Gal.Tra. Preceeding the fourth exon of α-LA gene there are two $(TG)_n$ repeats, $(TG)_{21}$ and $(TG)_{24}$ which have a potential of forming Z-DNA structures, and have been implicated in genetic recombination, rearrangement and regulation of gene expression. We have proposed that the entire fourth exon of the primordial gene of α-LA and lysozyme, according to split gene hypothesis, might have been replaced by a new region of DNA which partly coded for new a functional unit of a protein that had the property to interact with Gal.Tra.

We are currently determining the regulatory elements of the mammary α-LA gene which are involved in its expression. Some of these studies have shown

that the gene sequence which carries the $(TG)_n$ repeats and a part of the fourth exon which codes for the C-terminal domain of α-LA, acts as an enhancer in CAT assays suggesting that the nucleotide region which codes for the functionally important region of α-LA also carries some of the elements of gene regulation. Based on this observation, we propose a general hypothesis that the nucleotide region of a gene which codes for a functionally important domain of a protein may have some elements of gene regulation. Since the expression of α-LA gene in the mammary gland is known to be regulated by several polypeptidal and steroidal hormones and the gene contains several potential steroid receptor binding sequences, we are currently delineating the regions and the nucleotide sequences which bind these receptors and various other protein factor(s) which might be involved in the regulation of the expression of this gene.

We have undertaken the isolation and characterization of Gal.Tra. cDNA clone so that we can study Gal.Tra. gene expression during developmental differentiation and neoplastic transformation, and understand the modulation of the Gal.Tra. activity which generates specific cell surface antigenic determinants. Commercially available bovine Gal.Tra. was repurified and subjected to tryptic digestion. Some of the tryptic peptides were sequenced. A mixed 21-mer and 27-mer nucleotide probes, corresponding to two different peptides, have been used as hybridization probes to screen a bovine cDNA library constructed in the Okayama-Berg expression vector from the lactating bovine mammary gland poly (A)+RNA Analysis of the Gal.Tra. cDNA clone shows that the messenger RNA for the Gal.Tra. is much longer than expected from the molecular weight of the protein. The complete cDNA sequence and the encoded protein sequence is being analyzed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09016-02 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Protein Synthesis During Oncogenic Transformation

PRINCIPAL INVESTIGATOR *(List other professionel personnel below tha Principal Investigetor.) (Name, title, laboratory, end institute affiliation)*

Herbert L. Cooper	Chief, Cell. & Mole. Phys. Sec.	LTIB, DCBD, NCI
Richard Braverman	Chemist	LTIB, DCBD, NCI
Robert Bassin	Chief, Biochem. of Oncogenes Sec.	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	0.6	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We compared 2-dimensional electropherograms of newly synthesized proteins from lines of NIH/3T3 cells transformed by a variety of retroviral oncogenes, from cellular revertant lines, and from a line (433.3) which expresses the v-ras oncogene in response to corticosteroids. We detected 7 proteins whose synthesis was strongly suppressed in cell lines transformed by each of the six retroviral oncogenes we studied, and whose production was fully or partially restored in two cellular revertant lines. Suppression of two of these proteins was also correlated with the initial appearance of morphological alteration during steroid-induced oncogene expression in 433.3 cells. These proteins (p37/4.78 and p41/4.75) were identified as tropomyosins, a group of at least 5 cytoskeletal proteins. Transformation by the papova viruses, SV-40 and polyoma, caused no suppression of synthesis of these tropomyosins. This indicates that suppression of tropomyosin synthesis is not a nonspecific response by cells to being forced to grow with the transformed phenotype, but is specifically associated with oncogenesis by diverse retroviral oncogenes. The results are consistent with the hypothesis that the different biochemical processes initiated by expression of structurally diverse retroviral oncogenes may converge on a limited number of common targets, one of which is the mechanism which regulates the synthesis of tropomyosins.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

This project investigates the biochemical mechanisms which may link oncogene expression to neoplastic cellular transformation. Atlhough it is presumed that the protein products of oncogenes modify cellular metabolism in such a way as to induce neoplastic growth, the mechanism by which this occurs remains unknown. Our approach rests on the prediction that the activity of oncogene products will modify the synthesis, posttranslational modification, subcellular location, or metabolic fate of one or more normal cellular proteins whose function is essential for normal growth control.

To study this question, we have used 2-dimensional gel electrophoresis to study the proteins synthesized by a number of cell lines derived from NIH/3T3 cells and transformed by several oncogenic viruses. We have also examined cellular revertants (lines C-11 and F2), which exhibit a normal growth pattern but which retain the oncogene and its product, and a line of the long terminal repeat of the Mouse Mammary Tumor Virus joined to the Harvey v-ras oncogene. This line grows normally in the absence of corticosteroids. On addition of corticosteroids, 433 cells are induced to transcribe the v-ras gene, which results in transformation and permits detailed biochemical studies of events occurring during the transformation sequence. This material was made available by Dr. R. Bassin.

Although the gene sequences and protein products of the various well-studied oncogenes differ widely, the transformed phenotype produced by their expression shows great uniformity. Our study was designed, therefore, to emphasize the detection of events which might be common to the action of many oncogenes in the production of the transformed phenotype. In this way we hoped to focus on major cellular regulatory events whose derangement contributed to neoplastic transformation and which might ultimately provide an approach for therapeutic intervention.

Methods Employed:

Long-term cultured cell lines were maintained by standard cell culture techniques. Proteins were radiolabeled in intact cells by incubation with labeled amino acids. Preparations for analysis of proteins were obtained from whole cells and from subcellular fractions prepared by detergent lysis, mechanical homogenization in hypotonic solutions, sonication or N_2-cavitation followed by density sedimentation and ultracentrifugation. Proteins were analyzed by one and two-dimensional electrophoresis in polyacrylamide gels. Proteins were visualized in gels by staining with Coomassie Blue or silver, or by radiofluorography or radioautography. Tryptic peptide maps were obtained by trypsin digestion of single protein spots cut from two-dimensional gels which were hydrated, digested with electrophoresis/chromatography. Tryptic fragments were visualized by radioautography. Quantitation of synthesis or labeling of specific proteins was performed by direct measurement of radioactivity by liquid scintillation counting of solubilized single spots cut from gels.

Major Findings:

Initially we compared the relative rates of synthesis of proteins in control NIH/3T3 cells with those of the DT line of NIH/3T3 transformed by the Kirsten v-ras oncogene, using the technique of two-dimensional polyacrylamide gel electrophoresis. We noted 27 proteins whose synthesis was modified in the transformed cells to an extent which was considered significant and consistently detectable. To control for the possibility that altered synthesis of specific proteins in the DT cell line might arise through selection during prolonged cultivation, or through other culture-related artifacts, 5 other cell lines independently transformed by Ki v-ras were examined. Of the 27 proteins originally selected for study, synthesis of 10 of them was consistently inhibited in all lines transformed by Ki v-ras. We detected no cellular proteins whose synthesis was consistently increased in all these lines.

Synthesis of these 10 proteins was examined in cell lines transformed by 5 other related and unrelated retroviral oncogenes (Ha v-ras, fms, mos, fes, and src) in order to detect proteins whose synthesis might be suppressed as a general concomitant of neoplastic transformation. We found that 5 of these proteins were supressed in all lines examined, while 3 additional proteins were suppressed in all but src-transformed cells. Thus, 5 and possibly 8 proteins were evidently suppressed as a common event in expression of a variety of retroviral oncogenes.

To examine the relationship of suppression of synthesis of these proteins to the onset of oncogene-related transformation, we studied their synthesis in the 433 line of cells which express Ha v-ras and become transformed in response to corticosteroids. We found only 2 proteins (designated p37/4.78 and p41/4.75) of the selected group whose synthesis was suppressed coordinately with the onset of microscopically visible transformation when 433 cells were exposed to corticosteroids.

To determine whether suppression of synthesis of any of these proteins was, in fact, related to expression of the transformed phenotype we examined their synthesis in two cellular revertant lines derived from the DT transformed line. These revertants express the non-transformed phenotype in culture and are non-tumorigenic in nude mice, although they retain the oncogene and continue to express its product. Examination of proteins synthesized by these cells showed restored synthesis of 7 out of 8 of the candidate proteins in one revertant and 5 out of 8 in the other. Thus, synthesis of at least 5 of the proteins suppressed by expression of several oncogenes appears to be correlated with cell growth characteristics.

Two proteins of particular interest emerge from the foregoing experiments, namely p37/4.78 and p41/4.75. These proteins were suppressed by expression of all retroviral oncogenes studied, were the only proteins of the candidate group suppressed early during the appearance of the transformed phenotype, and were restored to normal levels of synthesis in both revertant lines studied. Upon analysis of subcellular fractions, we found p37 and p41 to be prominent components of the cytoskeletal fraction. Three

of the other proteins whose synthesis was restored in at lest one revertant also were associated with the cytoskeleton. Thus, 5 of 7 proteins under consideration as common targets of oncogene action appear to be cytoskeletal in location.

Of the known cytoskeletal proteins, the family of tropomyosins are reported to have M_r and pI in the range of p37/4.78 and p41/4.75. We therefore compared the characteristics of p37 and p41 with those reported for tropomyosins. In addition to the characteristic M_r and pI, p37 and p41 were resistant to denaturation by heat and lacked tryptophan, both known attributes of tropomyosins. Coelectrophoresis of NIH/3T3 cell extracts with purified tropomyosin from rabbit muscle showed very similar electrophoretic mobility in two dimensions. An antiserum to chick muscle tropomyosin reacted strongly with components migrating identically to p37 and p41. Finally, tryptic peptide maps of the immunoprecipitated proteins and the proteins observed in gels of NIH/3T3 cell lysates showed these proteins to be identical.

To determine whether suppression of tropomyosin synthesis was common to transforming modalities other than retroviral oncogenes, we examined the synthesis of p37 and p41 in NIH/3T3 cells transformed by the papova viruses, SV-40 and polyoma. Cells transformed by these agents have growth characteristics indistinguishable from those transformed by retroviruses. In these cells, we found no suppression of synthesis of p37 and p41. Thus, suppression of synthesis of these tropomyosins is not an invariable accompaniment of transformation in NIH/3T3 cells, but may be limited to transformation by retroviral oncogenes. This finding is evidence against the possibility that suppression of tropomyosin synthesis may be a cellular adjustment secondary to being forced to grow with the transformed morphology, since papova virus transformed cells exhibiting the identical morphology do not show this change.

Thus, we have shown that the two proteins which emerged from our analysis as the most promising common targets of retroviral oncogene action in the production of neoplastic transformation are tropomyosins.

Significance to Biomedical Research and the Program of the Institute:

Our results suggest a possible biochemical basis for the action of retroviral oncogenes in causing neoplastic transformation. The observations suggest that the biochemical pathways initiated by the expression of retroviral oncogenes may converge on the mechanism which regulates the synthesis of tropomyosin. Suppression of synthesis of tropomyosin may have disruptive effects on the cytoskeletal structure, which has long been known to be deranged in transformed cells. Our studies provide a possible biochemical basis for these structural changes, thereby contributing to basic understanding of the neoplastic process.

Proposed Course of Research:

Future plans for this project have been designed to address the following questions:

1. Is specific suppression of tropomyosin synthesis sufficient to induce all or even part of the trasnformed phenotype in NIH/3T3 cells?

2. Regardless of the role played by suppression of tropomyosin synthesis in transformation, this suppression is a clue to the biochemical consequence of oncogene expression and to cellular regulatory mechanisms. By what biochemical mechanism does oncogene expression cause suppression of tropomyosin synthesis?

These questions will be attacked by a molecular genetic approach, which will involve development of probes to TM mRNAs and genomic structures. Attempts will be made to manipulate levels of TM synthesis and to correlate these levels with appearance of components of the transformed phenotype.

Publications:

Cooper, H.L., Feuerstein, N., Noda, M., and Bassin, R.H. Suppression of tropomyosin synthesis: a common biochemical feature of oncogenesis by structurally diverse retroviral oncogenes. Molec. and Cellular Biol. (In press), 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09019-01 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cytoskeletal Proteins in Human Neoplasms

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Herbert L. Cooper	Chief, Cell. & Molec. Physiol. Section	LTIB, DCBD, NCI
Richard Braverman	Chemist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	0.8	0.9

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The synthesis and content of tropomyosins and other cytoskeletal proteins in various human tumor cell lines and tissues will be investigated in order to determine whether synthesis of these proteins is suppressed in human neoplasms as it is in NIH/3T3 cells transformed by oncogenic viruses. Tropomyosins synthesized by various tissue types will be identified by 2-dimensional polyacrylamide gel electrophoresis on the basis of molecular weight, isoelectric point, cytoskeletal localization, absence of tryptophan and immunological reactivity. Expression of tropomyosins in tumor cells of these types will then be evaluated. Synthesis of other cytoskeletal proteins will be evaluated in the same preparations. The following systems will be studied: lymphoid cells, myeloid cells, colon carcinoma, bladder carcinoma, and mammary carcinoma.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

Cytoskeletal derangement associated with expression of many retroviral oncogenes in NIH/3T3 cells may be related to suppression of synthesis of the tropmyosins, p37 and p41. However, transformation by papova viruses, which has also been reported to be associated with cytoskeletal derangement, does not cause suppression of these proteins. Thus, various transforming modalities may produce cytoskeletal derangement through different biochemical pathways. This project addresses the following questions:

How prevalent is suppression of tropomyosin synthesis among human tumors and experimental neoplastic cell systems?

In situations where tropomyosin synthesis is not disturbed, are there other biochemical derangements of cytoskeletal components?

Methods Employed:

Human peripheral lymphocytes were purified from heparinized whole blood (normal blood donors) by ficoll-hypaque sedimentation and plastic adherence. Long-term cultured cell lines were maintained by standard cell culture techniques. Lymphocyte growth was induced by exposure to mitogens (phytohemagglutinin, Con-A) when indicated. Proteins were radiolabeled in intact cells by incubation with labeled amino acids. Preparations for analysis of proteins were obtained from whole cells and from subcellular fractions prepared by detergent lysis, mechanical homogenization in hypotonic solutions, sonication or N_2-cavitation followed by density sedimentation and ultracentrifugation. Proteins were analyzed by one and two-dimensional electrophoresis in polyacrylamide gels. Proteins were visualized in gels by staining with Coomassie Blue or silver, or by radiofluorography. Tryptic peptide maps were obtained by trypsin digestion of single protein spots cut from two-dimensional gels which were hydrated, digested with trypsin, and analyzed by two dimensional thin-layer electrophoresis/chromatography. Tryptic fragments were visualized by radioautography and fluorography. Quantitation of synthesis or labeling of specific proteins was performed by direct measurement of radioactivity by liquid scintillation counting of solubilized single spots cut from gels.

Major Findings:

Our first objective was to establish a technique which could be used to identify tropomyosins among the whole-cell protein extracts of tissues and cultured cells of various developmental types. The approach was based on the following general characteristics of tropomyosins:

1. M_r and pI exhibited in 2-dimensional electrophoresis.
2. Cytoskeletal localization.
3. Absence of tryptophan.

Proteins showing these characteristics in cultured cells of particular types would be tested for reactivity with anti-tropomyosin serum to validate the identification. This information would be used to evaluate the synthesis of those tropomyosins in cultured human neoplasms or in freshly obtained fragments of tumor tissue. Previous pilot experiments had shown that acceptable 2-dimensional analyses of newly synthesized proteins in tissue fragments could be obtained by a modified organ culture technique.

This approach was applied to human peripheral lymphocytes and associated T- and B-cell neoplasms. In normal resting or growing human lymphocytes we identified two prominently synthesize tropomyosins of M_r ca. 35 K and pI ca. 4.75, differing by about one charge unit. These could be easily detected as prominently synthesized proteins in whole-cell displays of both resting and growing lymphocytes. Examination of whole cell protein patterns of established Burkitt lymphoma (B-cell) and T-cell leukemia cells showed no obvious alteration in synthesis of these tropomyosins in comparison with growing normal lymphocytes, nor did cytoskeletal preparations indicate any change in the representation of tropomyosins in the cytoskeleton.

Detailed quantitation of tropomyosin synthesis in this system is in progress, as well as study of additional lymphoma and leukemia cell lines. In addition, tryptic peptide maps of these proteins are being prepared to determine whether any consistent modifications of prmary structure of TMs may occur in neoplastic lymphoid cells.

The preliminary indications are that major quantitative changes in TM synthesis are probably not frequently associated with lymphoid neoplasia. It is therefore of interest to determine whether there is any indication of modification of other cytoskeletal components in this system. Comparison of newly synthesized cytoskeletal proteins of normal growing lymphocytes with those of B- and T-cell neoplasms showed prominent representation of two proteins ca. 20 Kda, pI ca. 4.72. These proteins were markedly reduced or absent in the lymphoma and leukemia cells examined. In another cell system (HL-60) we have previously identified human proteins having these electrophoretic characteristics as light chains of myosin, another major component of the cytoskeleton associated with microfilament function. Confirmation of this identification is currently in progress, as well as extention to additional lymphoid cell lines and evaluation of the status of the myosin heavy chain. If this modification proves to be consistent among lymphoid neoplasms, it will be important evidence for the generalized occurrence of specific biochemical lesions of cytoskeletal components in neoplasia, and raises the possibility of characteristic defects related either to cell type or to tumorigenic agent.

Significance to Biomedical Research and the Program of the Institute:

This study will help to determine whether the biochemical derangements associated with neoplastic transformation in experimental systems have

relevance to human malignancies. If such relevance is demonstrated, it may have both diagnostic and therapeutic significance in human oncology.

Proposed Course of Research:

The immediate plans are to complete the work in progress outline above. Particular attention will be paid to determine whether suppression of myosin synthesis is generally characteristic of human lymphoid neoplasms. If this identification is made unequivocally for available cultured cell lines, fresh clinical material will be sought. If indicated, a major long term effort will be undertaken to determine the biochemical basis for suppressed myosin synthesis. Molecular genetic approaches similar to those described for tropomyosin in Project 1 are envisaged.

An intermediate term goal will be evaluation of levels of tropomyosin and other cytoskeletal proteins in chronic and acute myelogenous leukemia. The question of the evolution of CML into AML has important clinical implications. If consistent differences in the synthesis of cytoskeletal proteins are detected between these cell types, it may prove possible to detect early stages of the CML to AML conversion, which may have therapeutic implications.

In the longer term we plan to examine a number of other established cell lines derived from human neoplasms. These include bladder carcinoma, colon tumors, mammary tumors, and lung tumors. It may also be possible to use a western blot technique, with appropriate immunological reagents, to make these determinations. A panel of monoclonal reagents to TMs is being developed and if these prove suitable, they may be used in collaboration with members of Dr. Schlom's group to study TM expression in human tumor sections by immunohistological techniques.

Another long term goal is the investigation of the biochemical functions of specific cytoskeletal components found to be modified in neoplasia. Clarification of the role of microfilaments and other cytoskeletal structures in normal nonmuscle cells with be part of that investigation, and may eventually provide insight into the role of those elements in the neoplastic process. Of particular interest are the structural and functional relationships between cytoskeletal elements and cell surface receptors for growth regulatory reagents, such as epidermal growth factor, platlet-derived growth factor, interleukins, and tumor growth factors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09006-03 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical Events in Phorbol Ester Effects on Normal and Tumor Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Herbert L. Cooper	Chief, Cell. & Molec. Phys. Section	LTIB, DCBD, NCI
Nili Feuerstein	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(If any)*

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.6	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Phosphorylation of proteins during response of cells to treatment with phorbol ester (PMA) was studied. In HL-60 promyelocytic leukemia cells arrest and differentiation after PMA exposure are associated with rapid phosphorylation-dephosphorylation of proteins pp17 and pp27. Cell-free studies suggest that this may involve the activation and cooperation of two classes of protein kinase, calcium-phopholipid-dependent kinase and cAMP-dependent kinase. Significantly, enhanced phosphorylation of pp17 and pp27 was found only in cell lines where PMA caused growth arrest and differentiation. The effect was minimal in cells where increased protein phosphorylation occurs. Elevated phosphorylation of class-I HLA molecules was documented, together with evidence suggesting association of HLA in a complex with myosin and actin and implicating modification of HLA as a component of platelet activation.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

This project represents the conclusion of a study carried out in this section over the past three years. It is based on the fact that cultured human promyelocytic leukemia cells (HL-60), when treated with tumor-promoting phorbol esters, abruptly cease to grow and then differentiate into nondividing monocyte-like cells. This behavior is equivalent to reversal of the neoplastic process, and is therefore of great interest. This project has characterized some of the early changes in protein synthesis and in protein phosphorylation associated with these events, in an effort to provide a biochemical basis for the observed changes in growth and function.

Methods Employed:

Human peripheral lymphocytes were purified from heparinized whole blood (normal blood donors) by ficoll-hypaque sedimentation and plastic adherence. Erythrocytes, monocytes and platelets were obtained from the same preparations. Long-term cultured cell lines were maintained by standard cell culture techniques. Lymphocyte growth was induced by exposure to mitogens (phytohemagglutinin, Con-A) when indicated. Proteins were radiolabeled in intact cells by incubation with labeled amino acids or inorganic ^{32}P. Preparations for analysis of proteins were obtained from whole cells and from subcellular fractions prepared by detergent lysis, mechanical homogenization in hypotonic solutions, sonication or N_2-cavitation followed by density sedimentation and ultracentrifugation. Proteins were analyzed by one and two-dimensional electrophoresis in polyacrylamide gels. Proteins were visualized in gels by staining with Coomassie Blue or silver, or by radiofluorography. Tryptic peptide maps were obtained by trypsin digestion of single protein spots cut from two-dimensional gels which were hydrated, digested with trypsin, and analyzed by two-dimensional thin-layer electrophoresis/chromatography. Tryptic fragments were visualized by radioautography and fluorography. Quantitation of synthesis or labeling of specific proteins was performed by direct measurement of radioactivity by liquid scintillation counting of solubilized single spots cut from gels.

Major Findings:

Initial studies characterizing phorbol ester-induced growth arrest and differentiation, and the attendant modifications in synthesis of specific proteins have been published. Attention was then turned to the initial biochemical response of the cells to the phorbol ester signal. Since phorbol ester binding to cells is known to activate Ca-phospholipid dependent protein kinase (C-kinase) in several systems, our study focussed on early phosphorylation events. Our results will be briefly summarized.

Within 15 minutes of exposure to phorbol myristate acetate (PMA), enhanced phosphorylation of two cytosolic proteins (pp17-20 and pp27, pI ca 5.5)

occurs. This enhancement is part of an increase in both phosphorylation and dephosphorylation specifically affecting these proteins, particularly pp17. Trifluoroperazine, which is known to inhibit C-kinase, completely blocked both the phosphorylation response as well as the growth arrest-differentiation of HL-60, suggesting a close relationship between the early phosphorylation events and later effects on growth and differentiation. The known relationship between PMA binding and C-kinase activation suggested that these events might be initiated by activation of C-kinase. Cell-free studies of protein kinase activities in this system suggested that, although C-kinase may be activated as an initial event in the response sequence, it is probably not directly responsible for the phosphorylation of pp17. cAMP-dependent protein kinase (A-kinase) was able to phosphorylate pp17 directly very efficiently. However, peptide mapping showed that A-kinase phosphorylated sites which differed from those phosphorylated on pp17 in the intact cell after PMA treatment. Thus, neither A- nor C-kinase alone appears to be responsible for pp17 phosphorylation, suggesting the existence of another unidentified protein kinase or of poorly understood interactions among the known kinases. The results suggest the existence of a sequence of interactions among kinases, perhaps initiated by activation of C-kinase.

The PMA-induced phosphorylation of pp17 was examined in a variety of cell systems in addition to HL-60. In A431 epidermoid carcinoma cells, where PMA also inhibits cell growth, pp17 was again strongly phosphorylated. However, in NIH/3T3 fibroblasts, JB-6 mouse epidermal cells, and human peripheral lymphocytes, where PMA has a mitogenic effect, little effect on phosphorylation of pp17 was observed. This suggests that increased phosphorylation-dephosphorylation of pp17 may play a role in the normalization of cell growth in those systems where binding of the PMA receptor (which may be equivalent to activation of C-kinase) results in inhibition of cell growth and/or differentiation.

In a related study, we have shown that activation of human platelets by phorbol esters is associated with increased phosphorylation of class I HLA molecules. Although platelets are one of the richest sources of HLA, the role of these molecules on platelets is unknown. Our observation provides the first direct evidence that these molecules may play a chemical role in the processes of platelet function. The observations support the idea that HLA molecules have significance beyond the immune system, perhaps indicating a more general role in cell-cell and cell-environment interactions to which cells respond by altered activity.

Significance to Biomedical Research and the Program of the Institute:

This study provides information about the biochemical events involved in response of human cells to powerful tumor promoting substances. In addition, the results bear on the question of return of differentiated activity to tumor cells which have become de-differentiated as part of the neoplastic process. Understanding of the biochemical background of such changes may be of great importance in future progress in cancer prevention and treatment.

Proposed Course of Research:

This project was carried out primarily by a post-doctoral fellow who has completed her training and has moved to a new position. Although many important questions remain to be answered in this system, evaluation of priorities in the context of the available resources, space and personnel led to the decision to terminate this project.

Publications:

Feuerstein, N., Sahai, A., Anderson, W., Salomon, D. and Cooper, H.L. Differential phosphorylation events associated with phorbol ester effects on acceleration versus inhibition of cell growth. Cancer Res. 44: 5227-5233, 1984.

Feuerstein, N., Monos, D., and Cooper, H.L. Phorbol ester effect in human platelets is associated with enhanced phosphorylatin of class I HLA antigens. Biochem. Biophys. Res. Commun. 126: 206-213, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00944-23 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Total Metabolism of Cancer Cachexia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Seoras D. Morrison Research Physiologist LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. Jeffrey Norton, Surgery Branch, NCI

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular and Molecular Physiology Branch

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The project concerns the causes and mechanisms of the nutritional depletion and general deterioration of the cancerous host, known as cancer cachexia. The object is to find ways of blocking or reversing the cachectic effects of cancer so that the cancer patient would become more accessible and less vulnerable to anti-cancer therapies. Insulin administered in both early (mild) and late (severe) cachexia increases voluntary food intake and conserves body mass without accelerating growth of tumor, but also without prolonging survival. The insulin treatment was unable to reverse or prevent the astenic component of cachexia. The simple mass of large experimental tumors used to study cachexia distorts the apparent cachectic effects of tumors, leading to over-estimate of effects on food intake, depression and tissue depletion, and underestimate of asthenic effects. Depletion of skeletal muscle by tumor is proportional to general carcass depletion, but normal hypertrophic response to work survives.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

(a) Identification of functiuonal sites and causes of breakdown of control of food intake during tumor growth. (b) Definition of the conditions and limitations for use of insulin as a therapeutic maneuver to prevent cancer cachexia and anorexia. (c) Identification of circulating factors in plasma that induce anorexia and metabolic abnormality. (d) Development of conceptual models of control of food and water intake and energy and water exchange and their inter-relationships for normal animals and for the cancer cachectic process.

Methods Employed:

The methods of indirect, total, long-term calorimetry, operant conditional responses, continuous or programmed infusions into unrestrained animals, and long-term cross-circulation preparations. In vivo and in vitro tracer studies of substrate turnover and protein degradation and synthesis. Computer methods of numerical analysis of tumor growth, energy exchange and feeding and drinking patterns.

Major Findings:

Tumor-bearing rats treated with exogenous insulin in early stages of cachexia increased food intake and host weight, and in late, severe cachexia maintained food intake and host weight with no acceleration of tumor growth in either situation. Survival time was not altered. Insulin treatment did not reverse the astenic component of cachexia.

An inert lump was implanted subcutaneously and "grown" in a pattern that simulated the non-malignant mass characteristics of commonly used large experimental tumors. The systemic responses seen to this mass show that the gross systemic responses to malignant tumors of this size overestimate the malignant effects on food intake and bodyweight and underestimate and even reverse the malignant effects on energy expenditure and asthenia.

Depletion of skeletal muscle (plantaris and soleus) in tumor growth is proportional to general carcass depletion, but the hypertrophic response of skeletal muscle to imposed work is unimpaired by presence of tumor.

Preliminary studies on a parabiotic preparation with one half bearing a tumor indicates the existence and transferrability of a blood-borne cachexigenic factor.

Proposed Course of Research:

Work on control system and metabolic characteristics of cancer cachexia will continue. Further studies will be done to assess the possible clinical use of insulin in combatting cachexia; particularly, the composition of the body mass

Significance to Biomedical Research and the Program of the Institute:

The findings on control of feeding and on the mechanisms and possible modes of prevention of cancer cachexia should be utilizable in the development of effective methods for improving the nutritional condition of cancer patients and their accessibility and response to therapy. The section of the National Cancer Plan that the work most closely approximates is: Objective 6 (Develop the means to cure cancers and to retard the progress of cancers not cured). Approach 4 (Enhance the host's ability to eliminate or prevent further development of cancer). It is also immediately relevant to the 1974 Amendment to the Cancer Act (Collect...information respecting nutrition programs for cancer patients and the relationship between nutrition and cancer).

Publications:

Morrison, S.D. Synergistic stimulation of food intake by simultaneous insulin and cold. J. Appl. Physiol. 57: 28-33, 1984.

Morrison, S.D., Moley, J.F. and Norton, J.A. Contribution of inert mass to experimental cancer cachexia in rats. J. Natl. Cancer Inst. 73: 991-998, 1984.

Moley, J.F., Morrison, S.D. and Norton, J.A. Insulin reversal of cancer cachexia. Cancer Res. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04848-13 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Use of Flat Cellular Revertants to Study the Function of Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert H. Bassin	Chief, Biochem. Oncogenes Sect.	LTIB, DCBD, NCI
Wayne Anderson	Research Chemist	LTIB, DCBD, NCI
Piero Tagliaferro	Visiting Fellow	LTIB, DCBD, NCI
Nikhat Najam	Visiting Fellow	LTIB, DCBD, NCI
Herbert L. Cooper	Chief, Cell. & Mole. Phys. Sect.	LTIB, DCBD, NCI
Peter Fuhrer	Expert	LTIB, DCBD, NCI
David Salomon	Supv. Research Biologist	LTIB, DCBD, NCI
Yoon Song Cho-Chung	Chief, Cell. Biochem. Sect.	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Makoto Noda, Inst. Phys. and Chem. Res., Tokyo, Japan
Wen Yang, Oak Ridge Natl. Lab., Oak Ridge, Tennessee
Ephriam Racker, Cornell Univ., Ithaca, New York

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Biochemistry of Oncogenes Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	2.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have continued to characterize 2 flat cellular revertants, C-11 and F-2, which were originally derived from the DT line of Ki-MuSV-transformed NIH/3T3 cells. A third revertant, Clone 22 was isolated during the last year. The revertant phenotype can be transferred by transfection of purified DNA from clone 22 to recipient Ki-MuSV-transformed cells. These primary transfectants, after repeated biological cloning, exhibit a stable nontransformed phenotype but are resistant to challenge with both v-Ki-ras and v-Ha-ras. A rudimentary "restriction map" for the putative revertant gene was derived by quantitating the effect of 8 different restriction endonucleases on the activity of revertant DNA as measured by transfection of the revertant phenotype into the DT cell line. We have initiated studies designed to reveal the mechanism responsible for the revertant phenotype. The resistance of the C-11 and F-2 revertants to the toxic effects of ouabain, together with our previous ion transport data, suggested that these cells were altered in some aspect of ion transport. Initial results indicated that the activity of the enzyme Na^+,K^+ ATPase, the specific target of ouabain, was the same in the NIH/3T3, DT and revertant cell lines, both in the presence and absence of ouabain. Preliminary studies using ^{86}Rb led to the same conclusion. We are currently studying the other major ion transport pathways in the revertant cell lines. In a collaborative project with Dr. Cooper, we have analyzed extracts from normal, transformed and revertant cell lines by 2-dimensional gel electrophoresis. The synthesis of only 2 proteins, with molecular weights of 38,000 and 41,000, consistently change with the ras-induced transformed phenotype. These proteins are present in normal NIH/3T3 cells, disappear in ras-transformed cells, and reappear in revertants. The proteins have both been identified as forms of the cytoskeleton-associated tropomyosin family. Additional studies indicate that the loss of tropomyosin is confined to cells transformed by retroviruses.

GPO 914-918

Project Description

Objectives:

The long-range goal of this project is an understanding of the mechanism by which various retroviral oncogenes induce the process of cell transformation in vitro and in vivo. Our current goal is to identify the cellular "target" molecules which are modified by the action of retroviral oncogenes, particularly by ras, and thereby to begin to deduce the pathways by which these oncogenes act.

Methods Employed:

Standard cell culture techniques continue to be employed for the generation, characterization and biological cloning of revertant cell lines. Susceptibility of the revertants to infection and transformation is carried out both by polyethylene glycol-mediated cell fusion and by infection with our own stocks of retroviruses. Other techniques include the isolation of high molecular weight DNA, transfection and cotransfection by the calcium phosphate technique and detection of transformed, neomycin-resistant, revertant, HAT-resistant and other cell phenotypes by appropriate selection techniques. Cells are also analyzed for Na^+,K^+ ATPase activity and for other aspects of ion transport as necessary.

Major Findings:

The revertant phenotype can be transferred from the C-11, F-2 and Clone 22 revertant cell lines to suitable recipient cells by the calcium phosphate coprecipitation technique using high molecular weight DNA extracts. We have found previously that the DT cell line, which has a low background of revertants resulting from changes in the ras gene, is suitable for transfection of the revertant phenotype, and is at least as susceptible as the standard NIH/3T3 cell line to transfection with pSV_2Neo DNA. Flat colonies can be detected in DT cells cotransfected with pSV_2Neo and high molecular weight DNA extracted from the revertant cell lines. We have isolated one such primary transfectant, and our preliminary experiments indicate that this line is resistant to challenge with the ras oncogene and has a rescuable transforming virus, both properties of the original revertant line from which it was derived.

We have digested high molecular weight DNA from the revertants and have attempted to measure the effects of restriction endonucleases on the number of flat colonies generated by Clone 22 DNA. Although this is a rather crude assay, it appears that certain endonucleases destroy the ability of Clone 22 DNA to generate flat colonies, while others do not.

The basis for the selection method used in isolating the revertants was the higher toxicity of ouabain for the transformed DT cell line as compared to the control NIH/3T3 cell line. We have examined the Na^+, K^+ ATPase activity in NIH/3T3, DT and 2 of the revertant cell lines by direct enzymatic assay and have found, in our initial experiments, that the

ATPase activity from all 4 lines was similar both in the presence and absence of 1 mM ouabain. We are currently examining other major pathways involved in ion transport in order to detect any possible differences among these cell lines.

Proteins extracted from the revertant cell lines, and from several other normal and transformed lines which have been generated in this laboratory, have been analyzed by 2-dimensional electrophoresis in an attempt to define changes in the synthesis of cellular proteins associated with transformation by the ras oncogene. Only 2 changes, the loss of a 38,000 and a 41,000 dalton protein, were consistently associated with the ras-transformed phenotype. These proteins are both forms of tropomyosin, as identified by immunoprecipitation. Similar changes were found not only in ras-transformed cells but also in cells transformed by the retroviral oncogenes mos, fes, fms, sis, and src. Changes in tropomyosin synthesis were not found in cells transformed by the papovaviruses polyoma and SV40.

Significance to Biomedical Research and the Program of the Institute:

Tremendous strides are now being made in identifying the role of retroviral oncogenes and the protein products which they encode. In many cases, these oncogene-derived proteins appear similar to growth factors or to receptors for growth factors, and may transform cells by turning on cellular growth mechanisms at an inapproriate time or by over-stimulating the normal cellular pathways which regulate growth and other fundamental biological properties. Since the discovery that a significant proportion of human tumors may involve the participation of genetic elements closely related to retroviral oncogenes, the opportunity is at hand to identify the specific cellular "target" molecules with which oncogene products interact and to define in molecular terms the pathways by which this intereaction leads to cell transformation. However, the lack of suitable cellular mutants that have alterations in the pathways involved in the transformation process is a major deficiency in these studies. Our object has been to isolate and characterize such mutants so that studies on the mechanism of cell transformation will be facilitated. Even with our initial set of revertants, all of which appear to be the same or very similar functionally, we have identified a protein which may turn out to be an important factor in transformation by retroviral oncogenes and an indication that the mechanism of transformation by ras may be related to ion transport.

Proposed Course of Research:

We will attempt to clone the gene responsible for the revertant phenotype using either the ori-defective cos cell system with an ori$^+$ SV40 vector or another suitable vector. We will also attempt to make additional doubly transformed cells of human and mouse origin for use in transfection experiments. The ultimate object of this experiment is to identify the gene responsible for the revertant phenotype.

We are continuing an analysis of the effect of transformation by the ras gene on ion transport in normal, transformed and revertant cells. The Na^+, K^+ ATPase pathway (sensitive to ouabain) having been excluded on the basis of our current data, we are investigating differences in 2 other major pathways of ion transport, the Na^+/proton antiport and the furosemide-sensitive pathway, in a search for major differences associated with transformation.

We will attempt to isolate additional revertants that are different from the ones already isolated by varying the oncogene employed and the selection method. We are also attempting to isolate revertants from transformed human lines to fuse directly with human tumor cells.

Publications:

Bassin, R. H., Noda, M., Scolnick, E. M., and Selinger, Z.: Possible relationships among onc genes using flat revertants isolated from Kirsten Sarcoma Virus-transformed Cells. Cancer Cells 2: 463-471, 1984.

Salomon, D. S., Zweibel, J. A., Noda, M., and Bassin, R. H.: Flat revertants derived from Kirsten Murine Sarcoma Virus-transformed cells produce transforming growth factors. J. Cell. Phys. 121: 22-30, 1984.

Cooper, H. L., Feuerstein, N., Noda, M., and Bassin, R. H.: Suppression of Tropomyosin synthesis: A common biochemical feature of oncogenesis by structurally diverse retroviral oncogenes. J. Mol. Cell. Biol. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08256-06 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Retinoids and Hormones in Mediating Cell Growth and Differentiation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Wayne B. Anderson	Research Chemist	LTIB, DCBD, NCI
Thomas P. Thomas	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

D. Evain-Brion, Unite INSERM 188, Paris, France, G.R. Grotendorst, LDBA, NIDR, NIH
S.P. Nissley, Metabolism Branch, NCI, NIH

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Biochemistry of Oncogenes

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Exposure of F9 teratocarcinoma cells to retinoic acid (RA) induces differentiation to an endoderm cell type. An early event of RA action to promote differentiation of F9 cells is to sensitize these cells to cyclic AMP by elevating cyclic AMP-dependent protein kinase (cAMP-PK) activities. Recent studies have determined changes in the activity and subcellular distribution of cAMP-PK in response to RA treatment of three different embryonal carcinoma (EC) cell lines. Retinoid-induced differentiation of F9 and PCC4 cells gives rise to a parietal endoderm cell type, whereas, exposure of PC-13 cells to RA induces differentiation to visceral endoerm. In every case the levels of cAMP-PK activity, and of the R_{II} regulatory subunit, were increased with RA treatment of the undifferentiated stem cell populations. However, with exposure to RA the amount of the R_I regulatory subunit was increased in F9 and PCC4 cells, which differentiate to parietal endoderm, whereas the level of R_I was decreased in PC13 cells which differentiate into visceral endoderm. RA treatment of F9 cells also causes a time-dependent (2-5 days) increase in cytosolic protein kinase C (PK-C) activity. With partially purified rat brain enzyme, RA was shown to inhibit diacylglycerol, or phorbol ester, stimulated PK-C activity. In the absence of added lipids, however, RA stimulated PK-C activity. Conceivably, RA might antagonize the actins of diacylglycerol or tumor promoters on PK-C.

EC cells have been shown to be a useful model to study early events of embryogenesis. Previously we established that rat insulin-like growth factor-II (IGF-II) can support the growth and differentiation of F9 cells. Results indicate that EC-derived endoderm cells produce an IGF-like activity. This factor competes for [^{125}I] IGF-II binding to membranes. This IGF-like factor is biologically active as it stimulates [3H] thymidine incorporation into chick embryo fibroblasts. Biochemical and immunological data indicate that this factor is closely related, if not identical, to IGF-II. These findings suggest that IGF-II produced by endoderm cells, particularly visceral endoderm, may serve as an early embryonic growth factor.

Project Description

Objectives:

To elucidate how retinoic acid acts to modulate cell growth and differentiation and to determine the role of growth factors and hormones produced and secreted by embryonal carcinoma cells on the growth and differentiated of these cells.

Methods Employed:

Cell Culture, standard biochemical analysis of protein kinase activities, photoaffinity labeling, hormone binding studies, SDS-polyacrylamide gel electrophoresis and radiographic analysis, immunoprecipitation and radioimmunoassay.

Major Findings:

Changes in calcium and cyclic AMP levels, as well as in protein kinase activities, have been implicated in the regulation of cell growth, differentiation, and malignant transformation. Thus, studies have been carried out to determine changes in cyclic AMP-dependent protein kinase (cAMP-PK) and Ca^{2+}-dependent protein kinase C(PK-C) activities in response to retinoic acid (RA). Cyclic AMP has been shown to promote RA-induced differentiation to parietal endoderm, and an early event of RA action appears to be to sensitizing F9 cells to cyclic AMP by increasing cAMP-PK activity. Recent studies have been carried out to determine possible differences in RA modulation of cAMP-PK activities with treatment of three different stem cell lines to induce differentiation to either parietal or visceral endoderm. RA treatment of F9 and PCC4 cells, which differentiate to parietal endoderm, causes an increase in cAMP-PK activity and in the amounts of the R_I and R_{II} regulatory subunits of cAMP-PK, both in the cytosol and membrane fractions. Exposure of PC13 stem cells to RA, which differentiate to visceral endoderm, caused an increase in cytosolic, but a decrease in membrane, cAMP-PK activity. Quantitation of regulatory subunits showed a decrease in the amount of R_I with a significant increase of R_{II} both in the cytosol and particulate fractions. These data indicate that the RA-induced changes in cAMP-PK activity and subcellular distribution perhaps influence the type of differentiation undergone by the stem cell population. The amount of R_I increases in cells differentiating to parietal, and decreases in cells differentiating to visceral, endoderm. In every case the levels of R_{II} regulatory subunits are low in undifferentiated cells and increase prior to the onset of differentiation, perhaps relating to the growth arrest generally observed following RA addition.

Protein kinase C is dependent on Ca^{2+} and phospholipids and is further stimulated by diacylglycerol. Several hormones, growth factors, and oncogene products may enhance phosphatidylinositol turnover to generate diacylglycerol and thus activate PK-C. RA added directly to the reaction mixture was found to inhibit diacylglycerol stimulated rat brain PK-C in a concentration-dependent manner. However, when assayed in the presence of Ca^{2+} and phosphatidylserine alone (no diacylglycerol) the addition of RA stimulated the partially purified PK-C. This could be of importance in mediating some of the effects of retinoids,

particularily those which serve to alter cell proliferation and antagonize TPA-induced tumor promotion. However, relatively high concentrations of RA are required to provoke a direct effect on PK-C activity, indicating this may not be a physiological response.

The differentiation of mouse embryonal carcinoma (EC) cells has been shown to mimic the differentiation of the early mouse embryo. These cells thus offer an in vitro system to study biochemical and regulatory events involved in early embryogenesis. We have used these cells as a model system to identify possible hormones and growth factors which might play a role in regulating embryonic growth and differentiation. Previous studies have shown that undifferentiated F9 stem cells secrete immunoreactive calcitonin, whereas endoderm cells produce parathyroid hormone immunoreactive material. Studies also have demonstrated that F9 stem cells possess specific cell surface receptors for rat insulin-like growth factor (IGF-II) and that IGF-II can serve as a potent growth promoter of F9 cells. Present results indicate that Dif 5 cells (endoderm cells derived from F9 cells following extensive exposure to RA) produce an IGF-II-like factor. When growth medium conditioned by Dif 5 cells in chromatographed on Sephadex G-75 in 1M acetic acid two peaks of activity are observed which compete for specific [^{125}I]iodo IGF-II binding to PYS cells. The high molecular weight fraction (Mr ~ 8K) is biologically active as this fraction stimulates [^{3}H] thymidine incorporation into serum-starved chick embryo fibroblasts. Radioimmunoassay data indicate that the IGF-like activity produced by Dif 5 cells is more closely related to IGF-II than to IGF-I.

Other studies indicate that Dif 5 cells produce a second factor which competes with [^{125}I]iodo PDGF for binding to target cells. This PDGF-like factor acts as both a chemoattractant and mitogen for smooth muscle cells. These findings raise the possibility that the factors identified may contribute to the regulation of cell growth, migration, and differentiation during the early stages of embryonic development.

Significance to Biochemical Research and the Program of the Institute:

Teratocarcinomas are malignant tumors which are characterized by the presence of an undifferentiated stem cell type known as embryonal carcinoma (EC) cells. EC cells have the capacity, depending upon their environment, either to form tumors or to differentiate into normal cells. It has been suggested that malignant stem cells show continued proliferation because neoplastic conversion has negated the ability of normal signals to induce differentiation. Thus, it is of importance to understand the mechanism by which retinoic acid acts to serve as a signal for the differentiation of malignant stem cells to non-malignant endoderm cells, and to understand the mechanism by which retinoic acid and phorbol ester tumor promoters are antagonistic in their effects on cell growth and differentiation. Certain tumors and tumorigenic cell lines have been reported to produce hormones and growth factors similar to those identified to be secreted by EC-derived cells. Since EC cells have some properties similar to early embryonic stem cells, it remains to be established if the production of these factors by EC cells is a property in common with early embryo cells or with neoplastic conversion. The inappro-

priate expression of such factors in normal cells could lead to neoplastic transformation.

Proposed Course of Research:

Investigations will continue to determine the possible production of, and altered responsiveness to, specific hormones and growth factors which may be involved in mediating the growth and differentiation of mouse and human teratocarcinoma cells, as well as of normal mouse blastocysts and embryos. Studies are planned to isolate and further characterize the identified factors to establish their role in altering cell growth and differentiation, and to establish any possible realtionship to malignant transformational events. Investigations also will continue in an effort to elucidate the mechanism of retinoic acid action in regulating cell growth and differentiation, and in modulating membrane-associated activities. Emphasis will be placed on studies to determine the mechanism by which retinoic acid modulates cyclic AMP-dependent protein kinase and protein kinase C activities.

Publications:

Evain-Brion, D., Binet, E., Donnadieu, M., Laurent, P., and Anderson, W.B.: Production of immunoreactive calcitonin and parathyroid hormone by embryonal carcinoma cells: alteration with retinoic acid-induced differentiation. Dev. Biol. 104: 406-412, 1984.

Anderson, W.B., Thomas, T.P., Plet, A., and Evain-Brion, D.: Effect of retinoic acid on cyclic AMP-dependent and calcium phospholipid-dependent protein kinase activities: Counteraction to phorbol ester tumor promoter. In: J. Saurat and P. Elias (Eds.): Retinoids: New Trends in Research and Therapy. (In press).

Nagarajan, L., Anderson, W.B., Nissley, S.P., Rechler, M.M., and Jetten, A.M.: Production of multiplication stimulatory activity (rat IGF-II) by endoderm-like cells derived from embryonal carcinoma cells: Possible mediator of embryonic growth. J. Cell. Physiol. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09015-02 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Identification of Cellular Targets of Oncogene Products

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Wayne B. Anderson	Research Chemist	LTIB, DCBD, NCI
Robert Bassin	Chief, Biochem. Oncogenes Sec.	LTIB, DCBD, NCI
Thomas P. Thomas	Visiting Fellow	LTIB, DCBD, NCI
Harvinder Talwar	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

W. Farrar, Lab. Mol. Immunoregulation, NCI, FCRF, Frederick, MD; D. Klein, Lab. Develop. Neurobiology, NICHHD, NIH; A. Spiegel, Metabolic Diseases Branch, NIADDKD, NIH; S. Aaronson, Lab. Cell. Mol. Biol., NCI, NIH

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Biochemistry of Oncogenes

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland, 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	2.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous studies have established that phorbol ester tumor promoters bind to, and activate, protein kinase C (PK-C), suggesting a role for PK-C in transformation and tumor promotion. Current studies have been devoted to determining if PK-C activity is altered and regulated in cultured cells in response to growth factors and other hormones, as well as in transformed cells. In pinealocytes, there is a synergistic mechanism operative between alpha$_1$- and beta-adrenergic agonists to elevate cyclic AMP levels. Treatment of pinealocytes with PMA, and with the synthetic diacylglycerol 1-oleoyl, 2-acetyl glycerol (OAG) (both of which act through stimulation of PK-C) was noted to mimic the effects of alpha-adrenergic agonists to enhance beta-adrenergic stimulation of pinealocyte cAMP accumulation. Further, treatment of these cells with the alpha$_1$-adrenergic agonist, phenylephrine, caused a rapid redistribution of PK-C activity from cytosol to pinealocyte membranes, indicating activation of this enzyme. These results suggest regulatory interaction between PK-C and neurotransmitter-dependent stimulation of cAMP levels. Exposure of an established lymphocyte cell line (CT6) to interleukin-2 (IL-2) was found to cause a rapid and transient redistribution of PK-C activity from soluble to particulate fraction. Phorbol myristate acetate (PMA) treatment of CT6 cells induced a similar transposition of PK-C in an analogous manner with the exception that PMA-mediated PK-C transposition to the plasma membrane is not readily reversed. Other studies demonstrated that interleukin-3 treatment of FDC-P1 cells provoked a similar decrease in cytosolic PK-C activity concomitant with an increase in membrane-associated PK-C activity. Thus, changes in the activation and subcellular distribution of PK-C in response to growth factors, tumor promoters, and transformation factors, may play a critical role in regulating such cellular processes as cell growth, differentiation, and malignant transformation.

PHS 6040 (Rev. 1/84) GPO 814-918

Project Description

Objectives:

To investigate the biochemistry and cell biology of certain onc gene products with focus on the plasma membrane as site of action to alter cell proliferation and malignant transformation. This will include studies to elucidate the mechanism by which tumor promoters, growth factors, and oncogene products alter membrane-associated protein kinase C.

Methods Employed:

Cell culture, standard biochemical analysis of protein kinase activities, protein purification procedures including ion exchange chromatography and hydrophobic affinity chromatography, SDS polyacrylamide gel electrophoresis and radiographic analysis, immunoprecipitation, and immunoblotting.

Major Findings:

Although the physiological function of oncogenes and cellular homologues of retroviral oncogenes (proto-oncogenes) are still largely unknown, it is expected that the products are essential in regulating cell growth and/or cell differentiation. Studies have been aimed at characterizing regulatory interactions of the two major transmembrane signal transmission systems [adenylate cyclase and cAMP-dependent protein kinase; phosphatidylinositol (PI) turnover and protein kinase C (PK-C)] and to determine if these important cellular regulatory systems are targets of transformational events. In cultured cells the tumor-promoting phorbol esters produce numerous functional and biochemical changes closely resembling or mimicking those induced by growth factors and transforming viruses. A variety of hormones and growth factors interact with their cell surface receptors to stimulate PI turnover and generate diacylglycerol, a physiological activator of PK-C. Tumor promoters such as myristate, acetate (PMA) also can bind to and stimulate PK-C apparently by substituting for diacylglycerol. Other studies have established that activation of PK-C by PMA is associated with a redistribution of PK-C from cytosol to membrane.

The pinealocyte adenylate cyclase system is unique in that alpha$_1$-adrenergic activation potentiates, rather than inhibits, the effects of beta-adrenergic agonists to enhance cAMP accumulation. Treatment of pinealocytes with the synthetic diacylglycerol, 1-oleoyl, 2-acetyl glycerol (OAG) was observed to mimic the effects of alpha-adrenergic agonists to increase beta-adrenergic stimulation of cAMP levels. Exposure of these cells to PMA promoted the association of PK-C with the particulate fraction and also potentiated the effects of norepinephrine to increase intracellular cAMP concentration. In addition, PK-C is rapidly redistributed from the cytosol into membranes in response to treatment of these cells with an alpha$_1$-adrenergic agonist, phenylephrine. These findings suggest that activation of PK-C is involved in the alpha$_1$-adrenergic amplification of beta-adrenergic stimulation of cAMP accumulation in these cells, and indicate some regulatory interaction between these two transmembrane signalling systems. PK-C activation under these conditions prompts the selective phosphorylation of a 35K membrane protein. Additional studies are required to determine if this 35K protein is involved in mediating alpha$_1$-agonist effects on cAMP accumulation.

Interleukin-2 (IL-2) acts through a specific cell surface receptor to promote the growth and differentiation of antigen-sensitized lymphocytes as well as large granular lymphocytes that express natural killer cell activity. Addition of IL-2 to an established IL-2 dependent cell line (CT6) was found to provoke a rapid (maximal at 10 min) loss in cytosolic PK-C activity. This loss in activity is transient, however, as activity is recovered within 60 min. Concomitant with the loss in soluble PK-C activity there is a transient increase in the amount of PK-C associated with the membrane fraction. PMA treatment of these cells, on the other hand, caused a similar, but stable, redistribution of PK-C activity from cytosol to membrane. Evidence also has been obtained that activated PK-C phosphorylates the IL-2 receptor as identified by the antigenic Tac moiety. It remains to be established how this phosphorylation event might alter IL-2 receptor function.

Interleukin-3 (IL-3) is a member of a family of growth and differentiation peptides, referred to as colony-stimulating factors, which regulates haematopoiesis. Exposure of FDC-P1 cells to IL-3 also caused a rapid and transient activation, and redistribution of PK-C activity. With both IL-2 (CT6 cells) and IL-3 (FDC-P1 cells) the proliferative dose-response paralleled the interleukin concentrations required to increase membrane-associated PK-C activity. The interleukins probably act through a PI hydrolysis-dependent mechanism with the generation of diacylglycerol to activate PK-C. Thus, a common feature of growth regulation shared by extracellular growth peptides, tumor promoters, and certain transforming gene products is the activation of PK-C.

Significance to Biomedical Research and the Program of the Institute:

The mechanism through which protein(s) coded for viral and cellular oncogenes (onc genes) act to alter normal cellular processes such as growth rate and to induce malignant transformation remain to be elucidated. Phorbol esters are tumor promoters that can amplify the effects of a low dose of carcinogen or other transforming agent, apparently by acting primarily at the cell surface. Phosphorylation of membrane proteins is an initial event induced by growth factors, and the intracellular location of certain viral transformation protein kinases is at the plasma membrane. Thus, growth factor-, phorbol ester-, and transformation-mediated changes in the activity and subcellular distribution of protein kinase C may be central to the regulation of cell growth and tumor promotion, and in events leading to the malignant transformation of cells. Results of these studies will lead to a better understanding of the control of cell proliferation and of the biochemical events critical to the expression of the transformed phenotype, and may provide new information for strategies of chemoprevention and chemotherapy.

Proposed Course of Research:

Studies will continue to determine if the phosphatidylinositol (PI) turnover-protein kinase C (PK-C) transmembrane signal transmission system serves as a target of certain cellular and viral oncogene products in growth regulation and tumor initiation and promotion. Changes in the subcellular compartmentalization of PK-C influenced by growth factors, tumor promoters, and transformation factors may alter association of this kinase with important

protein substrates. Studies also will continue to establish the regulatory importance of membrane association-dissociation of PK-C and to determine the mechanism, and regulation, of binding of PK-C to membranes with isolated components. Monoclonal and polyclonal antibodies to PK-C will be generated to facilitate these studies. Attempts also will be made to identify specific endogenous membrane proteins phosphorylated in response to tumor promoters and transformation factors, and to determine how these phosphorylation events might relate to membrane activities known to be modified by viral transformation such as monovalent cation transport and the adenylate cyclase system.

Publications:

Feuerstein, N., Sahai, A., Anderson, W.B., Salomon, D.S., and Cooper, H.L.: Differential phosphorylation events associated with phorbol ester effects on acceleration versus inhibition of cell growth. Cancer Res. 44: 5227-5233, 1984.

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Gopalakrishna, R., and Anderson, W.B.: The effects of chemical modifications of calmodulin on Ca^{2+}-induced exposure of a hydrophobic region: Separation of active and inactive forms of calmodulin. Biochim. Biophys. Acta 844: 265-270, 1985.

Farrar, W.L., Thomas, T.P., and Anderson, W.B.: Interleukin 3 activation of protein kinase C: Altered cytosol-membrane subcellular distribution. Nature (In press).

Sugden, D., Vanceuk, J., Klein, D.C., Thomas, T.P., and Anderson, W.B.: Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. Nature (In press).

Farrar, W.L., and Anderson, W.B.: Interleukin 2 stimulation of protein kinase C plasma membrane association. Nature (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05148-06 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mammary Tumorigenesis in Inbred and Feral Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert Callahan	Chief, Oncogenetics Section	LTIB, DCBD, NCI
Renato Mariani-Constantini	Visiting Associate	LTIB, DCBD, NCI
Chantal Escot-Theillet	Visiting Fellow	LTIB, DCBD, NCI
Robert Bassin	Chief, Biochem. of Onco. Sec.	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. Christine Kozak, LVD, NIAID, NIH
Dr. Michael Potter, LG, DCBD, NCI, NIH
Dr. Carlo Croce, Wistar Institute, Philadelphia, PA

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Oncogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In previous studies we have identified the chromosomal location of Unit I and Unit II endogenous MMTV genomes of BALB/c mice. We have now determined the chromosomal location of the Unit II endogenous MMTV genome using mouse/hamster somatic cell hybrids and genetic crosses between mouse strains. The Unit II MMTV genome is located on chromosome 6. Analysis of (C3H/OuJ x Czech II) x Czech II backcross mice revealed 1% recombinantion between the Unit II MMTV genome and the immunoglobulin kappa chain locus. The gene order with respect to other genetic loci on chromosome 6 is: centromere-Unit II MMTV/kappa chain gene-craf-cK-ras-2.

Earlier studies of tumor incidence in pedigreed breeding colonies of M. cervicolor popaeus (line D) and M. musculus musculus (Czech II) demonstrated that mammary tumor development is associated with a chronic infection by an MMTV related virus. Examination of line D mammary tumor cellular DNA by restriction enzyme analysis led to the following observations: (1) each restricted tumor cellular DNA contained new MMTV related fragments that are not present in normal liver cellular DNA, (2) 53% of the pregnancy independent tumors contained a rearranged int-1 locus as compared to 7% with an altered int-2 locus, and (3) in pregnancy dependent tumors the int-1 locus was affected in 33% of the cases whereas the int-2 locus was unaffected. These results point to a correlation between the activation of the int-1 locus and pregnancy independent mammary tumors.

We have previously demonstrated common host-viral junction fragments which do not correspond to either int-1 or int-2 in MMTV induced mammary tumor cellular DNA of Czech II mice. Recombinant DNA clones of one common host-viral restriction fragment has been obtained. Using unique host flanking sequences in this recombinant clone as a probe, we have been able to: (1) identify another mammary tumor with an insertion of MMTV in this region of the cellular genome, and (2) show that this new locus (designated int-3) is on chromosome 17.

Project Description

Objectives:

To identify the cellular genes at risk in MMTV and chemical carcinogen induced mouse mammary tumors and to determine the consequence of their activation or alteration in mammary tumorigenesis.

Methods:

Retroviral related sequences were detected in restriction endonuclease digested recombinant and cellular DNAs by the Southern transfer-blot hybridization technique. Recombinant clones containing MMTV related sequences were isolated from libraries of cellular DNA in Charon lambda phage. The pedigreed breeding colonies of feral mice have been described in Callahan et al., Proc. Natl. Acad. Sci. U.S.A. 79: 4113-4117, 1982.

Major Findings:

I. Endogenous MMTV of Inbred and Feral M. musculus domesticus.

We have previously shown using mouse/hamster somatic cell hybrids and genetic crosses between mouse strains that the Balb/c endogenous MMTV genomes designated Unit I and Unit II are located respectively on chromosomes 16 and 12. In our present studies, a 4% discordinancy was observed between retention of chromosome 6 and the presence of the 8.3 and 6.6 kbp MMTV related EcoRI fragments in 24 mouse/hamster hybrid DNAs examined. The immunoglobulin kappa chain genes, as well as the K-ras-2 and c-raf proto-oncogenes have each been mapped to chromosome 6. Since the C3H/OuJ strain of mice also contains the endogenous Unit II MMTV genome, we have monitored cellular DNAs from (C3H/OuJ x Czech II) x Czech II backcross mice, for the segregation of restriction fragment length polymorphisms (RFLP) of each of these genes. Cellular DNAs from seventy-four backcross mice were examined. One recombinant was observed between the kappa chain gene and Unit II MMTV. There was 8.7% and 31% recombination frequency between Unit II MMTV and respectively, the c-raf and K-ras-2 proto-oncogenes. Since the K-ras gene has been localized to the distal portion of chromosome 6, the gene order is centromere-Unit II MMTV/kappa chain gene - c-raf - K-ras-2. The fact that the three BALB/c endogenous MMTV genomes are located in the vicinity of each of the immunoglobulin loci and that plasma cell tumors frequently contain 12:15 and 6:15 chromosome translocation may be pertient to the observed expression of MMTV RNA in these tumors.

The available inbred strains of mice are derived from mice from several different locals in the United States and other parts of the world. We were interested in determining the frequency with which these strains contain common endogenous MMTV genomes. Our experimental approach was to use unique host restriction fragments flanking the Units II and III endogenous MMTV genomes as probes. EcoRI restricted cellular DNA from twelve inbred strains were tested. Unit II MMTV was present in all strains except NZB. The NZB strain was developed from mice trapped in New Zealand. Unit III was present less frequently, however again, the distribution of the strains and their geographical origin was wide spread.

To more fully address the question of the distribution and genetic diversity of endogenous MMTV genomes in M. m. domesticus, we, in collaboration with Dr. M. Potter (NCI), examined local populations of this mouse species from the Delmarva Pennisula. Mice were trapped from farms in 6 different areas by Dr. Potter and his colleagues. Random mice were selected from each populations for analysis. Cellular DNA was restricted by EcoRI and analyzed by Southern blot hybridization. The blots were hybridized with probes representing the MMTV LTR, gag, pol and env genes. The results of these experiments demonstrated the great diversity of sites within the cellular genome into which the MMTV genome can be inserted. In our study, none of the patterns of MMTV related restriction fragments appeared similar to those found in inbred strains of mice. In addition, two unexpected observations emerged from our study. Evidence was obtained which demonstrates that among individuals of a given deme there is extensive heterozygosity for the endogenous MMTV genomes. In the JJD population no two mice sampled had exactly the same pattern of endogenous MMTV genomes, even though some were siblings. In addition, mice lacking endogenous MMTV genomes were frequently encountered. Second, the vast majority of the endogenous MMTV genomes observed in the feral mice either lacked the gag or gag-pol genes, or contained only the LTR element.

II. Evidence for Common Integration Regions for Infectious MC-MTV in M. cervicolor popaeus Mammary Tumor Cellular DNA.

Attempts to transmit the M. cervicolor popaeus milk borne MC-MTV or tumor associated MC-MTV to the BALB/c or NIH Swiss strains of M. musculus by foster nursing or injection of concentrated virus have not been successful. At the molecular level, one characteristic of retroviral infection is the presence of additional integration proviral genomes in infected cellular DNA which are not observed in cellular DNA from uninfected tissue. We have compared matched sets of restricted normal liver and mammary tumor DNA from individual tumor bearing mice. Each restricted tumor cellular DNA contained new MMTV related fragments, that are not present in normal liver cellular DNA. In our view, this represents strong evidence for the presence of infectious MMTV in these mice. Our inability to transmit MC-MTV to M. musculus may reflect a limited host range or low infectivity of the virus.

The int-1 and int-2 loci were identified in mammary tumors of the C3H, BALB/cfC3H, and BR6 in bred strains of M. musculus. The int-1 locus of M. cervicolor popaeus is defined by overlapping 8.4 kbp BglIII and 8.0 kbp EcoRI restriction fragments which span 14 kbp of cellular DNA. The int-2 locus is defined by a 12 kbp HindIII and an adjacent 10 kbp EcoRI restriction fragment of cellular DNA. In the present study, 53% of the pregnancy independent tumors contained a rearranged int-1 locus as compared to 7% with an altered int-2 locus. In the pregnancy dependent tumors the int-1 was affected in 33% of the cases, whereas the int-2 locus was unaffected. Taken together these results point to a correlation between activation of the int-1 locus and the development of pregnancy independent mammary tumors. Although the int-2 locus is primarily affected in the pregnancy dependent tumors of BR6 mice, the sample size in the present study was too small for an accurate assessment.

Insertion of an MMTV genome into the int loci of M. musculus mammary tumor

cellular DNA leads to the unscheduled expression of flanking cellular sequences. M. cervicolor popaeus tumors which contain an MCMTV genome near the int-1 locus, also express a 2.6 kbp mRNA. This species of RNA is not present in mammary tumors in which the int-1 locus is unaltered. Since the MMTV LTR probe does not hybridize to the 2.6 kbp RNA, the mechanism of activation is probably similar to that proposed in inbred strains of mice.

III. Identification of a New int Locus in Spontaneous Tumors of M. musculus musculus (Czech II) Mice.

We have previously shown that MMTV induced mammary tumor DNA from three independent tumors contains common 7.2 and 7.7 kbp EcoRI host-viral junction fragments. In each case, the int-1 and int-2 loci were unoccupied. These results suggested to us that the 7.2 and 7.7 kbp EcoRI host-viral junction fragments define a new mammary tumor specific common integration region for MMTV. We have prepared a partial recombinant library of this tumor DNA and obtained a clone containing the 7.7 kbp EcoRI host-viral junction fragment. An XbaI-EcoRI fragment containing unique host flanking sequences was used as a probe to reexamine EcoRI restricted virus positive tumor cellular DNAs. Each of the tumor DNAs which contain the 7.7 kbp common host-viral junction fragment also contains a new 7.7 kbp fragment detected with the host flanking sequence probe. One other tumor DNA contains a new 12 kbp fragment which comigrates with MMTV LTR-gag containing fragment.

Studies with mouse/hamster somatic cell hybrids have shown that int-1 is located on chromosome 15 and int-2 is on chromosome 7. We have collaborated with Dr. Christine Kozak to determine the chromosomal location of the new Czech II locus. The results of these studies clearly show that the new locus is on chromosome 17. The results taken together demonstrate the existences of an int locus (designated int-3) in Czech II mammary tumor cellular DNA which is different than the int-1 or int-2 loci.

Significance to Biomedical Research and the Program of the Institute.

The contribution of the endogenous viral genomes in low incidence strains of mice to chemical carcinogen induced mammary tumors is not known. Recently, we and others have identified a novel 1.6 kb species of MMTV RNA in the BALB/c lactating mammary gland. This same RNA species is also expressed in all pristane induced plasma cell tumors and mammary gland hyperplastic outgrowth lines. This species of MMTV RNA is composed primarily of LTR related sequences. The LTR contains a long open reading frame capable of encoding a 26-30 K Dalton molecular weight protein. Knowledge of the chromosomal locations of the BALB/c endogenous genomes could lead to the development of substrains of Czech II mice (no endogenous MMTV) which contain individual BALB/c MMTV proviral genomes. It should then be possible to determine the contribution of these proviral genomes to the development of spontaneous and chemically induced mammary gland hyperplastic avelor nodules.

Our study of mammary tumorigenesis in M. cervicolor popaeus extends previous findings in inbred mice by: (1) demonstrating an association between MMTV induced rearrangement or activation of the int-1 and int-2 loci and mammary tumor development in a distantly related feral species of mice, and (2) acti-

vation of these cellular genes in mammary tumors does not appear to be limited to highly infectious laboratory strains of MMTV. Not all of the M. cervicolor popaeus mammary tumors (50%) contain insertions in the int-1 or int-2 loci. It is possible that some have occured outside the regions defined by the restriction enzymes used in this study. In our view a more likely possibility is that there are still other int loci which have not as yet been identified.

The Czech II mouse strains has proven useful in the identification of a new int locus in mammary tumor cellular DNA. The identification and future characterization of the int loci should provide a rational basis for developing an understanding of the cellular genes at risk in MMTV induced mammary tumorigenesis and the consequences of their activation.

Proposed Course of Research:

Our efforts over the next three years will initially focus on the molecular and biological characterization of the int-3 locus. The purpose of this study is to determine the nature of the genetic lesion caused by MMTV (Czech II) insertion mutation and the consequences of this event in mammary tumorigenesis. Other studies will focus on: (1) whether there are other as yet unidentified int loci or cellular oncogenes associated with MMTV induced mammary tumors in feral mice; and (2) the identification and characterization of the cellular genes which contribute to dimethylbenzathracine (DMBA) induced mammary tumorigenesis in Czech II mice. We also anticipate a limited effort to study the basis for MMTV expression in BALB/c mammary gland hyperplastic nodules and plasma cell tumors.

Publications:

Callahan, R., Drohan, W., Gallahan, D., D'Hoostelaere, L., and Potter, M.: A novel class of MMTV related DNA sequences found in all species of Mus including mice lacking the MMTV proviral genome. Proc. Natl. Acad. Sci. USA 79: 4113-4117, 1982.

Callahan, R.: Expression of mouse mammary tumor virus genes in Balb/c plasma cell tumors. In Melchers, F., Potter, M., and Weigert, M. (Eds.) Mechanisms of B cell Neoplasia Workshop at the Basel Institute for Immunology, Roche, Basel, Switzerland, 1983, pp. 92-98.

Callahan, R., Gallahan, D., and Kozak, C.: Two genetically transmitted BALB/c mouse mammary tumor virus genomes located on chromosome 12 and 16. J. Virology 49: 1005-1008, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04829-11 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Identification and Characterization of Human Genes Associated with Neoplasia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert Callahan	Chief, Oncogenetics Section	LTIB, DCBD, NCI
Iqbal Ali	Visiting Scientist	LTIB, DCBD, NCI
Renato Mariani-Constantini	Visiting Associate	LTIB, DCBD, NCI
Charles Theillet	Visiting Fellow	LTIB, DCBD, NCI
Toby Horn	Medical Staff Fellow	LTIB, DCBD, NCI
Robert Bassin	Chief, Biochem. of Oncogenes Sect.	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Drs. Ing-Ming Chiu and Stuart Aaronson, LCMB, DCCP, NCI;
Dr. Carlo Croce, Wistar Institute, Philadelphia, PA

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Oncogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

We have described a recombinant clone of human fetal liver cellular DNA (HLM-2) which contains sequences related to the MMTV genome. Further analysis of HLM-2 and four other MMTV related recombinant clones has shown: (1) the human MMTV gag-pol related sequences are organized in a manner consistent with that of a genetically transmitted proviral genome; (2) the human proviral genome contains a mosaic of sequences characteristic of different retroviral genera; (3) the HLM-2 proviral genome is representative of a large family (50 to 100 copies per cellular genome) of endogenous retroviral genomes; (4) there are approximately 1,000 copies of the LTR like elements in human cellular DNA; (5) this class of endogenous retroviral genomes has been intimately associated with several primate species during the course of evolution; and (6) expression of HLM-2 proviral related RNA has been detected in a primary breast tumor. Based on our current information, we conclude that the HLM-2 proviral genome does not represent the human analogue of MMTV, but instead, represents a novel class of primate endogenous retroviruses.

Project Description

Objectives:

To characterize at a molecular and biological level human cellular genes associated with the development of breast and colon neoplasia.

Methods Employed:

Retroviral and oncogene related sequences are detected in restriction endonuclease digested celluar DNA and recombinant DNA by Southern transfer-blot hybridization techniques. Poly A+ RNAs are electrophoretically separated on denaturing agarose gels, then blotted on to membrane filters for hybridization. Standard techniques are used to obtain recombinant clones of cellular and retroviral related DNA and to determine DNA nucleotide sequences.

Major Findings:

I. The Organization of MMTV Related Sequences in Recombinant Clones of Human Cellular DNA.

Recently, we have analyzed several more MMTV related human recombinant clones by restriction enzyme and heteroduplex analysis. Two relevant observations emerged from this study. First, the human MMTV related sequences defined in HLM-2 are members of a highly diverged family of retroviral related sequences in human cellular DNA. This was demonstrated by the large number of restriction site polymorphisms observed between different clones and by heteroduplex analysis. Each of the recombinant clones contained the repeat sequence observed in HLM-2. This repeat sequence shares no homology with the alu family of repetitive sequences and was always found flanking the MMTV gag-pol related sequences. Based on these and other results, we have speculated that the MMTV gag and pol related sequences in the human recombinant clones represent genetically transmitted or endogenous proviral genomes. The repeat sequences found in HLM-2 and the other MMTV related recombinant clones probably correspond to long terminal repeat (LTR) elements.

II. The Organization of MMTV Related Sequences in Human and Other Primate Cellular DNAs.

We have begun to assess the organization of HLM-2 proviral related sequences in human cellular DNA. Since the HLM-2 3.6 kbp EcoRI fragment shares the greatest homology with several different classes of retroviruses, we used this fragment to probe restricted cellular DNA from the MCF-7 human mammary tumor cell line using stringent blot hybridization conditions. Consistent with the restriction site polymorphism noted in the analysis of the recombinant clones, families of discrete restriction fragments containing sequences related to the HLM-2 3.6 kbp EcoRI fragment were detected. Significantly, the pattern of major EcoRI related fragments (3.6, 3.5, 2.9 and 1.9 kbp) is similar to that observed using the entire MMTV genome as a probe under low stringency blot hybridization conditions. The use of other restriction enzymes revealed the presence of major related cellular DNA fragments which are identical in size to those found in different human recombinant clones. This suggests that our collection of

MMTV gag-pol related human recombinant clones is representative of the major families of viral related restriction fragments observed in human cellular DNA. The frequency of these clones in the human recombinant DNA library (55 positive plagues/5 x 10^5 plagues tested) suggests that there are several copies of these sequences in human cellular DNA. Quantitative dot blot analysis with HLM-2 probes is consistent with 50 copies of the pol and 1,000 copies of the LTR sequences in human cellular DNA. This result raises the obvious possibility that a significant number of the LTR-like elements are not associated with retroviral related structural genes. To test this, we have rescreened the human fetal liver library with the human LTR as a probe. Three recombinant clones were picked which react with restriction fragments containing the LTR but not the viral related structural genes. These clones are currently being analyzed.

The large sequence diversity observed in the different MMTV related recombinant clones, suggests that in evolutionary terms, this family of retroviral genes was not recently introduced into the human germline. We have begun to assess the conservation of these retroviral related sequences in other primate species. EcoRI restricted cellular DNA from chimpanzee, rhesus and squirrel monkey each react under low stringency blot hybridization conditions with the human LTR and pol-env probes. The similarity in the pattern of related restriction fragments in chimpanzee and human cellular DNA is striking. When this blot was washed under stringent conditions, the intensity of the viral related fragments decreased in a manner expected for the evolutionary relationship of these primates to humans. These results suggest that this class of retroviral genomes has been intimately associated with primates throughout much of their evolution.

III. Expression of Human Endogenous Retroviral Related RNA in Human Normal and Tumor Tissues.

This study has recently been initiated. We have prepared poly A+ RNA from ten primary breast tumors, three breast tumor cell lines, two colon carcinoma cell lines, two normal fibroblast cell lines, a normal spleen and two placentas. The RNAs were electrophoretically separated on denaturing agarose gels. Blots of the gels were probed with labeled restriction fragments corresponding to different regions of the HLM-2 proviral genome. One breast tumor RNA contains 6.5 kb species which reacts with the HLM-2 3.6 kbp EcoRI pol-env restriction fragment. However, this RNA did not react with the HLM-2 LTR probe.

Significance to Biomedical Research and the Program of the Institute:

Relatively little is known about the human endogenous retroviruses. The results presented here define a new class of highly diverged endogenous retroviruses in human cellular DNA. The role which these viral related genes play in the etiology of neoplasia is unknown. However, the large copy number of LTR-like elements in human cellular DNA raise the possibility that they may be mobile insertion elements. Whether the LTR-like elements can act as insertion mutagen should be the focus of further study.

Proposed Course of Research:

Our aim in the near future is to complete the molecular and biological charac-

terization of this new class of endogenous retroviral genomes as well as the "solitary LTR" elements. These studies should lead to an understanding of whether they contribute to the development of neoplasia. In addition, we propose to further characterize the human MMTV env related sequences which we have detected in human cellular DNA. In the future, we plan to expand the scope of our studies on human breast and colon neoplasia to include the cellular proto-oncogenes and the human analogues of the murine int loci.

Publications:

Callahan, R., Drohan, W., Tronick, S., and Schlom, J.: Detection and cloning of human DNA sequences related to the mouse mammary tumor virus genome. Proc. Natl. Acad. Sci. USA 79: 4113-4117, 1982.

Chiu, I.M., Callahan, R., Tronick, S.R., Schlom, J., and Aaronson, S.A.: Major pol gene progenitors in the evolution of oncoviruses. Science 223: 364-370, 1984.

Callahan, R., Ali, I., Fetherston, J., Horn, T., and Schlom, J.: Human endogenous retroviral genes and their possible role in oncogenesis. Cell Tech. 12: 1103-1108, 1984.

Callahan, R., Chiu, I.M., Wong, J.F.H., Tronick, S.R., Roe, B., Aaronson, S.A., and Schlom, J.: A new class of genetically transmitted human retroviral genomes. Science (In Press).

Callahan, R., Chiu, I.M., Horn, T., Ali, I., Robbins, J., Aaronson, S.A., and Schlom, J.: A new class of human endogenous retroviral genes. In Rich, M. (Ed.): RNA Tumor Viruses in Human Cancer, (In Press).

Callahan, R., Ali, I., Fetherston, J., Horn, T., and Schlom, J.: The organization of mouse mammary tumor virus related sequences in human cellular DNA. In Varier, O.E., Gallo, R.C., and Stehelin, D. (Eds.): Retroviruses and Human Pathology, (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05190-05 LTIB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Monoclonal Antibodies Define Carcinoma Associated and Differentiation Antigens

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)*

Jeffrey Schlom	Chief	LTIB, DCBD, NCI
Patricia Horan Hand	Chemist	LTIB, DCBD, NCI
Ann Thor	Medical Staff Fellow	LTIB, DCBD, NCI
Raffaella Muraro	Visiting Fellow	LTIB, DCBD, NCI
Rose Tran	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)* P. Noguchi, Bureau of Biologics, FDA
D. Kufe, Dana Farber Cancer Institute
L. Liotta and C.N. Rao, Laboratory of Pathology, DCBD, NCI
J. Lundy, SUNY, NY

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.3	3.3	2.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies involve the generation and utilization of monoclonal antibodies (MAbs) to identify and characterize human carcinoma associated antigens and differentiation antigens of mammary and colonic epithelium. These MAbs are being used to better understand the cell biology and pathogenesis of human carcinomas, and to provide reagents for use in several aspects of the management of human carcinomas. These include: detection of occult tumor cells; further defining the degree of differentiation of "normal", dysplastic, and carcinoma cell populations; serum antigens assays; and radiolocalization of primary and metastatic carcinoma lesions in situ (and potentially therapy) using radiolabeled monoclonal immunoglobulins and fragments. These studies are divided into four areas of investigation: (I) The generation and characterization of an MAb that defines a novel tumor associated antigen (TAG-72); (II) The development and characterization of MAbs to a repertoire of epitopes on carcinoembryonic antigen (CEA) which are differentially expressed among carcinoma cell populations; (III) The generation and characterization of MAbs to proteins associated with metastatic cell populations, and (IV) The definition and characterization of breast and colon differentiation antigens.

GPO 914-918

Project Description

Objectives:

To generate and characterize monoclonal antibodies that are reactive with human mammary tumor associated antigens. These monoclonal antibodies may prove useful in the diagnosis, prognosis, monitoring of therapy, and eventual therapy of human carcinomas, as well as in the study of the biology of human carcinoma cell populations.

Methods Employed:

Hybridoma technology was used to generate monoclonal antibodies against human breast tumor metastases. The antibodies were screened in a solid phase radioimmunoassay for reactivity with extracts of mammary tumor metastases to various organs and corresponding normal tissue. The reactivity of the antibodies to cell surfaces was examined using a live cell RIA and by fluorescence activated cell sorting. The reactivity of these antibodies to tissue sections of primary tumors and metastases to the lymph node and distal areas as well as normal tissues was examined using the immunoperoxidase technique.

Major Findings:

I. A Monoclonal Antibody (B72.3) Defines the Distribution of a Novel Tumor Associated Antigen.

MAb B72.3 is a murine IgG_1 that was generated using as immunogen Protein A affinity purified membrane-enriched fractions of a breast carcinoma metastasis to the liver. Membrane-enriched fractions were prepared to increase the probability of obtaining MAbs reactive with cell surface antigens; these fractions were subjected to Protein A sepharose chromatography to remove endogenous human IgG that could conceivably mask the presence of putative tumor associated antigens (TAAs). Our laboratory has systematically relied on direct clinical materials for immunizations and initial screenings of MAbs, due to the possibility that breast or colon cancer cell lines may consist of cell populations that are not necessarily reflective of cells of actual primary or metastatic lesions. This hypothesis was set forth due to the high degree of selective pressure that is exerted in the establishment of these cell lines. This has indeed been shown to be the case for MAb B72.3, in that the reactive antigen is rarely expressed on carcinoma cell lines in culture. MAb B72.3 was initially selected by screening hybridoma culture supernatant fluids in solid phase radioimmunoassay (RIA) to extracts of biopsy material (i.e., positive reactivity to extracts of breast carcinomas and negative reactivity to extracts of a variety of normal adult human tissues). It was also shown to be negative for reactivity to mouse mammary tumor virus and other murine retroviruses, purified CEA and purified ferritin. MAb B72.3 has been shown in both immunoprecipitation and Western blotting experiments to react with a high molecular weight glycoprotein of greater than 10^6d termed TAG (Tumor Associated Glycoprotein)-72.

To further characterize the range of reactivity of MAb B72.3, the avidin-biotin-complex (ABC) immunoperoxidase technique was used on Formalin-

fixed tissue sections to define TAG-72 expression in a variety of malignant, benign and normal mammary tissues. In all tissues tested, frozen sections and Formalin-fixed sections gave similar results. Using 4 ug of purified IgG of MAb B72.3 per slide, the percent of positive primary breast tumors was 46% (19/41); 62% (13/21) of the metastatic lesions scored positive. Several histologic types of primary mammary tumors scored positive: these were infiltrating duct, infiltrating lobular, and comedo carcinomas. Many of the in situ elements present in the above lesions also expressed TAG-72. None of the six medullary carcinomas tested were positive. Approximately two-thirds of the tumors that showed a positive reactivity demonstrated a cell associated membrane and/or diffuse cytoplasmic antigen pattern; approximately five percent showed discrete focal staining of the cytoplasm, and approximately one-fourth of the reactive tumors showed an apical border or marginal antigen localization. Metastatic breast carcinoma lesions that were positive were in axillary lymph nodes, and at the distal sites of skin, liver, lung, pleura and mesentery. Fifteen benign breast lesions were also tested; these included fibrocystic disease, fibroadenomas and sclerosing adenosis. Two of these 15 specimens showed positive staining: one case of fibrocystic disease where a few cells in some ducts were faintly positive, and a case of intraductal papillomatosis and sclerosing adenosis with the majority of cells staining strongly. Monoclonal B72.3 was also tested against normal breast tissue from non-cancer patients and showed no reactivity. A variety of non-breast tissues were tested and were also negative or showed only faint patches of focal antigen expression; these included uterus, liver, spleen, lung, bone marrow, colon, stomach, salivary gland, lymph node and kidney. Hematopoetic malignancies, sarcomas and melanomas were also routinely negative.

MAb B72.3 was utilized to evaluate TAG-72 expression in other carcinomas with initial emphasis placed on human colon carcinoma. Using purified B72.3 IgG (10 ug/ml) and the ABC immunoperoxidase method on fixed tissue sections, 82% of colon carcinomas demonstrated some reactivity. The majority of the carcinomas that were positive had more than 50% of tumor cells present reacting. The cellular location of the reactivity also varied within a given tumor mass; luminal reactivity was often observed in glandular structures, with cytoplasmic staining in less differentiated structures. As in all the immunohistochemical analyses described, negative controls for the specificity of staining were used: parallel slides with buffer (instead of primary antibody), an isotype identical IgG (MAb MOPC-21), or normal murine IgG all gave negative results. Adenomas in patients with colon carcinoma were also examined for expression of TAG-72; only one of ten such lesions showed a positive reaction. This lesion was directly adjacent to a carcinoma and had less than 5% of cells positive. All other adenomas and adjacent normal epithelium in carcinoma patients were negative with one exception, in which less than 1% of the cells scored positive. Adenomas from patients without apparent carcinoma were examined; five of the 18 samples displayed MAb reactivity in a low percent of adenoma cells; none of the 18 lesions, however, showed reactivity similar to that observed in carcinomas. Malignant and benign colon lesions were then examined using ten-fold less (1 ug/ml) MAb B72.3. At this antibody dilution, 50% (8 of 16) of carcinomas showed a positive reaction; one of 10 adjacent adenomas showed a focal reactivity of less than one percent of adenomatous cells.

None of 16 adenomas from non-carcinoma patients and no normal epithelium from 19 carcinoma or adenoma patients reacted at this antibody dilution. Thus, a positive reactivity with MAb B72.3 at this dilution appears to be associated with malignancy.

II. MAbs Define a Repertoire of Epitopes on Carcinoembryonic Antigen (CEA) Differentially Expressed in Human Colon Carcinomas Versus Normal Adult Tissues.

Several studies have identified colon TAAs using both polyclonal antisera and MAbs. One group of colon tumor markers has been identified as oncofetal antigens; these are defined as molecules which are normally expressed during fetal development and re-expressed during neoplastic transformation. Of this class, CEA has been the most extensively studied. CEA has been described as a complex glycoprotein of 180,000 daltons which is highly expressed by both embyonic colonic mucosa and carcinomas of the gastrointestinal tract. There have been numerous reports, however, that indicate that "CEA" may actually be a family of isoantigens, and that anti-CEA antibodies may indeed differ dramatically in their reactivity.

There are several areas in which antibodies to CEA are now being used in the management of carcinoma patients; these include: (a) blood assays to monitor tumor recurrence, (b) immunohistopathologic analyses of tissue samples to detect or further characterize tumor cells, (c) in situ detection of carcinomatous lesions (either at primary sites, regional lymph nodes, or distal sites) using radiolabeled antibody, and (d) potentially, therapy. One of the major drawbacks of the use of anti-CEA antibodies for the above purposes has been the cross reactivity of these reagents with some apparently normal adult tissues, especially polymorphonuclear leukocytes (PMNS). Anti-CEA MAbs have been generated previously which cross-react with CEA related antigens on normal colonic mucosa, normal spleen, normal liver, normal lung, sweat glands, polymorphonuclear leukocytes, and monocytes. Thus, at present, the degree of differential expression of CEA in carcinomas versus normal tissues is not resolved. Some anti-CEA MAbs which are currently being used in radioimaging of colon cancer patients, have been shown to have at least some degree of reactivity to normal PMNs. Perhaps the most well characterized anti-CEA MAbs, in terms of range of reactivities to tumor and non-tumor tissues are those recently reported by Primus et al. and designated NP-1 through 4. MAb NP-1 was shown to react with polymorphonuclear leukocytes and MAbs NP-1 through 3 were shown to react with normal colon. The most highly selective anti-CEA MAb thus far reported in terms of differential reactivity for colon carcinoma versus normal adult tissues is MAb NP-4. One interesting finding of these studies, however, was that the NP-4 epitope is principally expressed on primary colon carcinomas and not in regional or distal metastases. Thus, it should have been emphasized that with all the studies reported characterizing numerous anti-CEA MAbs, to our knowledge, none exist (with the exception of NP-4) that have been extensively characterized as to differential reactivity with primary and metastatic colon carcinomas versus benign colon lesions and a wide range of normal adult tissues.

Previous attempts in our laboratory, using mammary tumors as immunogen, led to the development of anti-CEA MAbs and MAbs to CEA related antigens.

While some of these have proved very useful in experimental models, they may have limited clinical utility due to their reactivity to granulocytes (as detailed above for other anti-CEA MAbs). We thus set out to develop a series of anti-CEA MAbs that were extensively screened at a very early stage for lack of reactivity to spleens and granulocyte preparations known to be rich in CEA-cross reactive antigens. MAbs designated COL-1 through 15 have been generated and characterized and show a strong degree of selective reactivity for human colon carcinomas versus normal adult tissues. To prepare the MAbs, mice were immunized with extracts or membrane-enriched fractions of biopsy material from either primary or metastatic colon carcinoma lesions. The COL MAb, all of the IgG 1, 2a, or 2b subclasses react with purified CEA in solid phase RIA and by immunoblotting, but none reacted with human polymorphonuclear leukocytes or spleen preparations previously shown to be rich in CEA-related or cross-reactive antigens. When used in immunoblotting with colon carcinoma extracts, the COL MAbs detect only a 180K band, as opposed to the multiple bands observed with other anti-CEA MAbs. MAbs COL-1 through 15 could be divided into at least five groups based on their differential range of reactivities to the surface of colon carcinoma cells or other carcinoma cell preparations. The COL MAbs were tested via immunoperoxidase with a wide range of primary and metastatic colon carcinomas, benign or dysplastic colon lesions and 34 normal adult tissues. With few exceptions, the COL MAbs tested in this manner showed reactivity only to the primary and metastatic colon carcinomas.

These studies thus provide a well characterized repertoire of MAbs that are well suited for potential clinical trials involving the radiolocalization and possibly therapy of human colon carcinoma lesions. The fact that at least five epitopes are being recognized (as defined by differential expression among carcinoma cell populations) also provides the opportunity for testing the efficacy of cocktails of the COL MAbs toward these goals and to further study the structure of the CEA "molecule."

III. MAbs to Proteins Associated with the Metastatic Process.

The mechanism of tumor cell invasion appears to be a highly complex and multistage phenomenon. One necessary step in the invasive process is the movement of tumor cells through the extracellular matrix, which is composed of basement membranes and interstitial stroma and forms a barrier between tissues of different types. One of the components of the basement membrane is laminin, a glycoprotein with a molecular weight of approximately 10^6. A 50-fold greater binding of laminin to unoccupied receptors of human breast carcinoma cells versus normal and benign breast lesions has been reported. It has been proposed that laminin receptor may aid in the attachment of tumor cells to laminin in basement membranes thereby facilitating: (a) the exit of tumor cells from their tissue of origin, and (b) the penetration of tumor cells through other basement membranes, including those of blood vessels, to result in metastatic lesions. The existence of a laminin receptor was established when a high affinity receptor for laminin was first identified on the surface of the MCF-7 human breast carcinoma cell line. Since this initial observation, the presence of laminin receptor has been demonstrated on the surface of several normal and neoplastic tissues; these are rodent striated muscle, rodent macrophages, human monocytes,

as well as human breast cancer cells, metastatic murine BL6 melanoma cells, and murine fibrosarcoma cells. Laminin affinity chromatography studies have demonstrated that both the murine and human receptor molecules have a molecular weight of approximately 67,000-69,000.

In collaboration with Drs. Rao and Liotta (Pathology Branch, NCI) we have recently generated MAbs using purified human laminin receptor as immunogen (prepared by our collaborators). The MAbs bind to (a) the purified receptor coated on a solid phase; (b) isolated breast carcinoma plasma membranes; and (c) the surface of cultured MCF-7 human breast carcinoma cells by immunohistochemistry. Using immunoblotting, the MAbs recognize a single 67,000d protein among all the proteins extracted from breast carcinoma plasma membranes. The MAbs can be distinguished, however, in their ability to block binding of laminin to the plasma membrane receptor. Antibody LR1 inhibits virtually 100% of the specific binding of laminin to both isolated human breast carcinoma plasma membranes or living MCF-7 cells. In contrast, antibody LR-2 has no effect on laminin binding under identical conditions. Thus, two types of MAbs may recognize structurally distinct sites on the laminin receptor. These MAbs should thus be useful to dissect the biology and the molecular genetics of the laminin receptor.

It has been hypothesized that all endothelial and epithelial cells attach to basement membranes via the laminin receptor. Our results with MAb LR-3 (chosen because of its ability to efficiently bind Formalin-fixed tissue sections) define the expression of the laminin receptor molecule in a wide variety of cell types (endothelial and epithelial), and have substantiated these theories. We have shown that numerous epithelial and endothelial normal human cell types, virtually all adjacent to basement membranes, are positive for laminin receptor. We have also determined that pulmonary macrophages (cells not bound to basement membrane) were uniformly positive for laminin receptor expression. Recent findings suggest that macrophage cell surface laminin receptor may play an important role in metastatic tumor cell recognition; laminin receptor expression may facilitate attachment and emigration of the macrophage precursor cell (monocyte) through vascular basement membranes and other extracellular matrices during the inflammatory process. It has previously been shown that extracts of breast carcinomas express more exposed laminin receptor than normal breast tissues. These studies were carried out using ^{125}I-laminin and extracts of breast material. Our recent studies using MAb LR-3 now facilitate the detection of laminin receptor at the single cell level using immunohistochemical techniques. Differences among tumor cells in LR3 immunoreactivity may be related to absolute numbers of unoccupied, internalized or processed receptors. Thus, we have observed subpopulations of cells within some "benign" lesions expressing high levels of laminin receptor.

The biology of human mammary carcinoma and colon carcinoma is similar in many respects, including TAAs expressed on the cell surface. Our studies demonstrate for the first time the distribution of laminin receptor in human colon carcinomas and benign lesions. The degree of laminin receptor expression in the colon tissues closely parallels that observed with the

breast carcinomas and benign lesions. Not all colon carcinomas express detectable levels of the laminin receptor, but higher levels are clearly observed in malignant colon tumors versus benign lesions.

IV. Detection and Characterization of Breast and Colon Differentiation Antigens.

(A) Several MAbs have been described that are reactive with human mammary carcinomas. They can be classified into 3 groups on the basis of the immunogen used to generate the MAb; these are (a) breast tumor cell lines, (b) milk fat globule membrane, and (c) extracts of breast carcinoma metastases. Each of the MAbs thus far described, including those prepared by several different groups to milk fat globule membrane, appears to be unique with respect to percent of reactive mammary tumors, percent of reactive cells within tumors, location of reactive antigen within the tumor cell, and degree of reactivity with non-mammary tumors as well as normal tissues. In collaboration with Dr. D. Kufe (Dana Farber Cancer Center) we have defined a human breast antigenic determinant using a murine MAb (MAb DF3) prepared against a membrane-enriched fraction of a human breast carcinoma. The reactive antigen has a molecular weight of 290K and is detectable on the cell surface of human breast carcinoma cells using a live cell radioimmunoassay or fluorescence flow cytometry. Using immunoperoxidase staining of Formalin-fixed tissue sections, MAb DF3 distinguishes malignant and benign breast lesions. A cytoplasmic distribution of antigen has been observed with 40 of 51 (78%) breast carcinomas, as compared to one of 13 fibroadenoma or fibrocystic disease specimens. In contrast, reactivity of benign breast lesions with MAb DF3 primarily occurs along apical borders on ductules. These results reveal that the DF3 antigen is present on apical borders of more differentiated secretory mammary epithelial cells and in the cytosol of less differentiated cells. These findings suggest that the DF3 antigenic phenotype on human breast carcinomas might provide a correlate with the degree of differentiation.

Clinicopathologic parameters have been evaluated in predicting prognosis for breast cancer patients. Lymph node status is considered the single most important prognostic variable. Recent studies have suggested that estrogen receptor (ER) status, progesterone receptor (PgR) status, nuclear grade (NG) and histologic grade (HG) may also be prognostic indicators in lymph node negative and positive patients. ER positive tumors demonstrate evidence of tumor differentiation with low nuclear and histologic grades, while ER negative tumors exhibit poor tubule formation and anaplastic nuclei. Since reactivity of breast carcinomas with MAb DF3 may reflect degree of differentiation, we have assayed for the level of DF3 antigen as a correlate of primary tumor nuclear grade (NG), histologic grade (HG), and ER status. More DF3 antigen was present in breast carcinomas with less anaplastic nuclei (NG 1 and 2) as compared to tumors with anaplastic nuclei (NG 3) ($p = .002$). Similarly DF3 antigen presence was greater in better differentiated tumors (HG1 and 2) than in poorly differentiated tumors (HG3) ($p = .001$). We also demonstrated that the presence of the DF3 differentiation antigen correlated with estrogen receptor status. Twenty-two of 23 ER positive tumors were also DF3 positive; 6 of 23 ER negative tumors were reactive to MAb DF3 ($p = .001$). There was, however, no correlation between DF3 reactivity and absolute level of estrogen or progesterone receptor.

These findings support our hypothesis that MAb DF3 identifies a differentiation antigen of human breast epithelium. The DF3 antigenic phenotype may serve as an independent phenotypic marker with correlations to standard indicators of degree of differentiation and estrogen receptor status of infiltrating duct carcinomas of the breast, and should thus be evaluated as a prognostic indicator in breast cancer patients. The data also suggests that the use of MAb DF3 in histochemistry may be a useful alternative in assessing estrogen receptor status of small breast cancers where there is an insufficient amount of tumor present for biochemical assay of hormone receptor levels.

(B) Few, if any, colon differentiation antigens have been identified, and to date, no MAbs have defined such an antigen. We have recently generated an MAb (termed CDA) which may define a novel human colon differentiation antigen. MAb CDA was generated using a membrane-enriched fraction from a biopsy of a moderately differentiated human colon adenocarcinoma as immunogen. The reactive antigen was determined by Western Blotting and immunoprecipitation experiments to be a high molecular weight glycoprotein of greater than 400,000 daltons. As judged by immunohistochemical staining, MAb CDA reacted with the epithelium of the majority of specimens from normal, dysplastic and malignant colon, with greater reactivity observed in normal colon when compared to benign or malignant lesions. No significant reactivity was observed with normal mammary tissue and a variety of mammary carcinomas. Cell sorter analyses demonstrated a heterogeneous distribution of this antigen on the cell surface of the well differentiated colon adenocarcinoma cell line LS-174. Separation of antigen positive and antigen negative cells using MAb CDA and flow cytometric techniques, and inoculation of the two sorted cell populations into immunosuppressed rats resulted in tumors which differed markedly in their degree of differentiation. The CDA positive cells caused very well differentiated tumors, while the CDA negative population induced extremely undifferentiated tumors. Studies to further define the nature of this antigen as well as its biologic distribution and significance are in progress.

Significance to Biomedical Research and the Program of the Institute:

These studies have led to the generation of a series of monoclonal antibodies that are reactive with the majority of human mammary and colon carcinomas examined. These antibodies may eventually prove useful in the dignosis, prognosis, and treatment of human mammary and colon neoplasia, as well as in the study of the basic biology of human carcinoma cell populations.

Proposed Course of Research:

The high molecular weight glycoprotein (TAG-72), detected by MAb B72.3, has been shown to have a great deal of differential expression in mammary and colon carcinoma cells as opposed to benign counterparts and a variety of normal adult tissues. Future plans are to define the biochemistry of TAG-72 and to utilize MAb B72.3 to further define the expression of this tumor antigen in the pathogenesis of both colon and breast carcinomas. Preliminary studies indicate that TAG-72 is expressed at higher levels in ovarian carcinomas than in colon or breast carcinomas. We plan to determine if TAG-72 expression is correlated with malignant vs. benign lesions, and which type of "borderline" ovarian masses express this antigen. We have

recently been able to partially purify the TAG-72 antigen from human colon carcinomas. This will be used as immunogen to obtain "second generation" MAbs to TAG-72. The second generation MAbs will also provide a means for analyzing the use of cocktails of MAbs in radioimmunodetection and therapy studies, and will provide reagents to develop a double determinant RIA for TAG-72.

The COL MAbs recently generated can be divided into at least five groups based on biologic distribution of reactive determinants, i.e., the differential reactivities to carcinoma cell populations. The COL MAbs are currently being evaluated for their ability to be (a) radiolabeled with isotopes such as ^{131}I and ^{111}In, and (b) reduced to Fab, Fab' and $F(ab')_2$ fragments, without loss of immunoreactivity and subsequently radiolabeled. Those which can be both successfully fragmented and radiolabeled will be considered as candidates for use in in vivo detection and, using higher ^{131}I levels and coupling with alternative isotopes, perhaps radioimmunotherapy of colon carcinoma lesions.

Very few if any colon differentiation antigens exist and none have thus far been identified with MAb technology. Our recent studies with MAb CDA show that it reacts in immunoprecipitation and Western blotting of colon carcinoma cell extracts with an antigen of approximately 400,000d located on the tumor cell surface. We plan to learn more about the nature of the CDA molecule. Our recent RIA and immunohistochemical analyses have shown that the antigen is expressed on the vast majority of normal colonic epithelial cells and is expressed to a lesser extent on benign colon lesions and still less on colon adenocarcinomas. This has provided the first evidence that the antigen being detected by MAb CDA may be a differentiation antigen. Preliminary cell sorter analyses with MAb CDA support this hypothesis. We plan to continue our collaborative studies to further analyze the biology of the two cell populations defined by MAb CDA in fluorescence activated cell sorter analyses.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09017-01 LTIB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*
Oncogene Expression in Human Carcinomas

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Patricia Horan Hand	Chemist	LTIB, DCBD, NCI
Ann Thor	Medical Staff Fellow	LTIB, DCBD, NCI
Arnaldo Caruso	Visiting Fellow	LTIB, DCBD, NCI
Maureen O. Weeks	Chemist	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.9	1.4	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Monoclonal antibodies (MAbs) of predefined specificity have been generated by utilizing a synthetic peptide reflecting amino acid positions 10-17 of the Hu-ras^{T24} gene product as immunogen. When paraffin-embedded Formalin-fixed tissue sections and the avidin-biotin complex immunoperoxidase methods were used, the RAP (RA, ras; P, peptide) MAbs clearly defined enhanced ras p21 expression in the majority of human colon and mammary carcinomas. The majority of all abnormal ducts and lobules from fibroadenoma and fibrocystic disease patients were negative, as well as normal mammary and colonic epithelia examined.

We have used the RAP MAbs to define ras p21 protein expression in a spectrum of colonic disease states. Immunohistochemical analyses of individual cells within tissue sections reveal differences in ras p21 expression in colon carcinomas compared with normal colonic epithelium, benign colon tumors and inflammatory or dysplastic colon lesions. Our data suggest that ras p21 expression is correlated with depth of carcinoma within the bowel wall, and is probably a relatively late event in colon carcinogenesis.

We have recently developed a quantitative liquid competition RIA for ras p21 using MAb Y13259. By the use of a standard of pure recombinant ras p21, we are now able to detect p21 at fM levels, and to determine the number of molecules of p21 per cell. The concomitant use of this quantitative RIA and immunohistochemical analyses of normal, dysplastic and inflammatory disease states, as well as carcinomas, should lead to a better understanding of the role of the ras gene in the genesis of these lesions.

Project Description

Objectives:

The purpose of these studies is to generate and utilize monoclonal antibodies (MAbs) to the ras p21 oncogene and proto-oncogene products to determine the role, if any, of the ras gene in the etiology and pathogenesis of human carcinomas.

Methods Employed:

Hybridoma technology was used to generate MAbs. The reactivity of the MAbs was examined using techniques of immunohistochemistry and by solid phase and liquid radioimmunoassays.

Major Findings:

Several oncogenes have been associated with human carcinomas. Of these, the ras oncogene has thus far received the most attention. There are two mechanisms by which ras can mediate cellular transformation: (a) via a point mutation at position 12 or 61 of the genome. This altered gene can be detected by DNA transfection assays using the NIH-3T3 murine fibroblast indicator system, or by restriction endonuclease analysis, or (b) via enhanced expression of the normal or proto-onc form of the ras gene. This latter mechanism has been demonstrated by the findings that proto-onc ras, when ligated to an active retroviral long terminal repeat (LTR) promoter, can transform NIH-3T3 cells. While the demonstrations that several human carcinomas contain point-mutated ras were quite definitive, point-mutated ras is actually found in only a small percentage of certain tumors of a given cell type. For example, to date no point-mutated ras has been observed in any human breast adenocarcinoma tested, and only approximately 10% of human colon carcinomas contain this altered gene form. In view of the fact that most oncogenes involved in the transformation process act via enhanced expression of the proto-onc form of ras p21 and previously published studies that enhanced expression of the proto-onc forms of both rat ras and human ras can mediate cell transformation, we undertook studies to analyze and quantitate levels of the 21,000d protein (p21) ras gene product in human breast and colon carcinomas. Our goal was to analyze ras p21 expression from a tumor biology and tumor pathogenesis point of view in an attempt to gain some insight into the role of ras gene expression in the multistep process of carcinogenesis. To accomplish this, we first set out to develop and/or utilize MAbs to ras p21 with which we could both (a) define ras p21 expression at the single cell level, and (b) accurately quantitate ras p21.

(a) Generation of MAbs. Since the DNA sequence and thus the corresponding protein sequence of the human ras proto-onc and oncogene are known, and it has been shown that synthetic peptides (when properly coupled) can serve as immunogens, we set out to develop MAbs to synthetic peptides reflecting amino acid positions 10-17 of the ras genome. This region was chosen so that one could potentially obtain MAbs that (a) react with both the proto-onc and point mutated form of ras p21, and/or (b) react preferentially with the point-mutated form. MAbs were initially screened for reactivity to peptides reflecting the position 12 point mutated ras p21 (termed Hu-ras^{T24}, from the T24 human bladder carcinoma

cell line from which the gene was derived) and Hu-ras^{Ha} or proto-onc form, i.e., endogenous human ras^{Ha}. Murine MAbs designated RAP (RAs Peptide) 1 through 5, of the IgG_{2a} subclass, were selected for further study with emphasis being placed on MAb RAP-1 and 5. In solid-phase RIAs the RAP MAbs reacted with both the Hu-ras^{T24} and the Hu-ras^{Ha} peptide. Initial attempts to immunoprecipitate native p21 from extracts of transformed cells proved unsuccessful. This was not unexpected, because the determinants detected by the RAP MAbs have been predicted to be internally located in the native p21 molecule. Immunoblotting experiments in which extracts were treated with $NaDodSO_4$/ mercaptoethanol were thus used to demonstrate binding to p21. The RAP MAbs showed low levels of binding in solid-phase RIAs to undenatured cell extracts of the T24 human bladder carcinoma cells and T24 DNA-transfected NIH-3T3 cells; no binding to NIH-3T3 cells was detected. As discussed above, one would not predict efficient binding of these MAbs to native ras p21; extracts were therefore treated with 10% buffered Formalin or 2% buffered glutaraldehyde in an attempt to alter the configuration of p21 and thus expose the determinants detected by the RAP MAbs. Formalin treatment of extracts of either the T24 human bladder carcinoma cell line or the MCF-7 human mammary carcinoma cell line appreciably enhanced detection of ras p21 by the RAP-MAbs. Formalin did eliminate, however, the binding of MAb Y13-259 (another anti-ras p21 MAb henceforth referred to as Y-259) to T24 cells. We thus set out to determine if the RAP MAbs could be used in Formalin-fixed human tissue sections to identify ras p21 expression. Formalin-fixed tissue sections have advantages over frozen sections including (a) better preservation of cellular detail and histologic architecture and (b) better availability, so that a broader spectrum of tissues can be examined.

(b) Analysis of Human Mammary Carcinomas for ras p21 Expression. Sections from 30 Formalin-fixed infiltrating ductal carcinomas (IDC) from 30 patients were examined for ras gene activation with each of several RAP MAbs (RAP-1, -2, and -5), with all the MAbs yielding similar results. Twenty-seven of 30 (90%) of the IDCs scored positive. Dilution experiments with purified IgG of the RAP MAbs were carried out as a "semiquantitative" measure of the relative amounts of ras p21 among tissues. These MAb dilution experiments demonstrated that most "positive" mammary carcinomas contain cells positive for cytoplasmic ras expression at MAb endpoint dilutions 32- to 320-fold higher than those dilutions required for cells in the vast majority of benign lesions. An arbitrary separation among the carcinomas exists, however, between those tumors scoring $\leq$5% of carcinoma cells positive (11/30) and those with $\geq$20% of tumor carcinoma cells scoring positive (19/30). By this criterion - i.e., $\geq$20% of tumor cells scoring positive-only 2 of the 21 benign breast lesions (0/11 fibrocystic disease and 2/10 fibroadenomas) scored positive. It is interesting to note that the two fibroadenomas with elevated ras gene expression were from two patients with multiple fibroadenomas (7 tumors from 2 patients prior to age 24). The one of 11 fibrocystic disease specimens examined with 10% of cells scoring positive for ras expression was from a patient with a clinical history of severe chronic mastitis and histologic diagnosis of fibrocystic disease with focal chronic inflammation. However, the vast majority of all abnormal ducts and lobules from fibroadenoma patients and fibrocystic disease patients were negative. Normal lobules and ducts from breasts of patients with fibroadenoma and fibrocystic disease were also routinely negative for ras gene expression.

In most primary mammary carcinomas, a heterogeneity in the number of tumor cells expressing ras was observed. Several mammary tumor metastases from five patients with ras-positive primary mammary tumors were assayed with MAb RAP-1 and also showed some degree of heterogeneity of ras gene expression within individual metastatic masses.

(c) Detection of ras p21 in Colon Carcinoma. Sections of Formalin-fixed colon carcinomas were assayed for Hu-ras p21 content using purified immunoglobulin preparations of MAb RAP-5 and the ABC immunoperoxidase method. Initial studies using a 40-fold range of purified antibody and serial sections of tumors demonstrated four of the six colon carcinomas clearly positive for ras p21 expression. The cell type expressing ras in these sections was the malignant epithelium, with the number of positive carcinoma cells per tumor mass ranging from 20% to 90%; the number of reactive cells could be reduced in a linear fashion by decreasing the concentration of the RAP antibody used. Colonic stroma and smooth muscle were negative for ras p21. Normal colonic mucosa from carcinoma patients (when seen within the same section containing carcinoma cells) was either negative for reactivity with RAP-5 at all immunoglobulin concentrations used, or contained less than 1% of normal epithelial cells expressing ras p21. Normal colonic epithelium from a non-cancer patient also contained less than or equal to 1% ras p21 positive cells at all RAP-5 concentrations. Four benign colon tumors (two tubular and two tubulovillous adenomas) were also assayed for ras p21 expression using RAP-5 concentrations and assay conditions identical to those described above. The tumor cells from all four polyps assayed were either negative for RAP MAb binding or contained only a few cells positive over the 40-fold range of antibody concentration used.

To further define if biological correlations exist in ras p21 expression, colon carcinomas, benign colon tumors, inflammatory lesions, and 18 specimens of normal colon from non-cancer patients were examined for reactivity with MAb RAP-1. Of the 47 carcinomas, 45 contain some ras positive carcinoma cells; however, a distinction could be observed with 23/47 of the tumors from individual cancer patients containing greater than 20% of tumor cells expressing ras p21 at the antibody concentration used. In contrast, 4 of 18 normal colon samples from non-cancer patients contain $\leq$1% positive cells with the remainder scoring negative. Several types of benign and inflammatory colon lesions were also assayed. Of these 38 lesions, 36 are either negative for ras p21 expression or contain $\leq$5% of epithelial cells scoring positive, with the remaining two lesions containing 10% positive cells. We conclude that with the RAP antibody concentration and conditions used for these assays, enhanced ras p21 expression can be observed (using >20% cells positive as an arbitrary criterion), in 23 of 47 colon carcinomas, as opposed to none of 38 benign colon tumors, inflammatory colon specimens, or 18 normal colonic tissues (all from different patients)

Antigenic heterogeneity has been demonstrated in most human colon carcinomas using monoclonal antibodies directed against several distinct tumor associated antigens. We have found that most colon carcinoma specimens also vary in the percent of tumor cells within a given tumor mass expressing ras p21. We examined five metastatic lesions from two colon carcinoma patients whose primary tumors were positive for ras p21 expression; all five metastatic lesions also displayed antigenic heterogeneity.

There is much evidence that many colon carcinomas arise from "premalignant" disease states such as adenomas, familial adenomatous polyposis, or ulcerative colitis. If such a multistep process is involved in colon carcinogenesis, it would be of interest to determine where in this process enhanced ras p21 expression is detected. Upon examination of invasive carcinoma in a pseudopolyp of an ulcerative colitis patient, the mildly dysplastic epithelial cells of the pseudopolyp react weakly with the RAP antibody. In contrast, adjacent cells (which are more severely dysplastic and/or carcinoma in situ as well as invasive carcinoma) express comparatively higher levels of ras p21. These studies provide evidence that ras p21 activation, at least in these carcinomas, may be a relatively late event in the transformation process.

The biologic degree of malignancy (potential for metastasis) of colon carcinoma correlates with the depth of tumor invasion through the bowel wall (from the muscularis mucosa to the serosa). This invasion may occur via the infiltration of individual tumor cells through the stromal elements and musculature of the bowel wall or via vascular or lymphatic invasion; these tumor cells are believed to subsequently divide and form individual cell clusters with various degrees of glandular organization depending on the histologic degree of tumor differentiation. We identified specimens from three colon cancer patients in which benign colonic mucosa, as well as the depth of invasion, could be clearly evaluated within a single tissue section. In these samples, normal colonic epithelia were either negative or expressed extremely low levels ($\leq$1% of cells) of Hu-ras p21. Superficially invasive carcinoma cells showed intermediate levels of Hu-ras p21, whereas deeply invasive tumor cells showed the highest levels of ras p21 in the tumor mass. Thus, in each of the several cases in which the depth of individual malignant tumor cells and glandular structures could be evaluated, enhanced Hu-ras p21 expression correlated with depth of tumor invasion.

(d) Development of a Liquid Competition RIA for ras p21. Our studies involving the use of the RAP MAbs and immunohistochemical analyses of tissue sections indicate an enhanced expression of ras p21 in malignant mammary and colon carcinomas. These studies include experiments in which purified IgG of RAP MAb was diluted and assayed on serial sections of different malignant and benign tumors, and normal tissues as a means of determining relative antigen titers; these experiments, however, must be considered "semiquantitative" at best. To accurately quantitate the amount of ras p21 within cells in culture or in biopsy materials, we have recently developed quantitative liquid competition radioimmunoassays (RIAs) for ras p21. Both assays use MAb Y-259 a rat IgG MAb previously shown to immunoprecipitate the native form of both rat and human ras p21. As a standard for the sensitivity and specificity of the assays, we have obtained bacterial extracts of recombinant Hu-ras^{T24} and Hu-ras^{proto} as well as control bacterial extracts. Both ras preparations compete completely and with identical slopes in the direct binding assay. We have also obtained purified recombinant ras p21 (at least 95% pure) as a standard for quantitation. Based on the amount of purified ras p21 required for competition in this RIA, we can thus determine the moles of ras p21 per ng of competitor protein as well as the number of molecules of ras p21 per cell in virtually any sample which competes with identity in this assay.

Significance to Biomedical Research and the Program of the Institute:

Several oncogenes have been associated with human carcinomas. Of these, the ras oncogene has thus far received the most attention. There are two mechanisms by which ras can mediate cell transformation: (a) via a point mutation of the genome. This altered gene can be detected by DNA transfection assay using the NIH 3T3 murine fibroblast indicator system, or by restriction endonuclease analysis. While several human carcinoma lesions have been shown to contain point mutated ras, this lesion is usually found in only a small percentage of tumors of any given cell type, or (b) enhanced expression of the normal or proto-onc form of ras.

Our studies reinforce the hypothesis that elevation of ras p21 expression is involved but may be but one event of many in the multistep process of tumor cell initiation, promotion, and progression of colon and breast cancer. A quantitative liquid competition RIA for ras p21 has also recently been developed. By the use of a standard pure recombinant ras p21, we are now able to detect p21 at fM levels, and to determine the number of molecules of p21 per cell. The concomitant use of this quantitative RIA and immunohistochemical analyses of normal, dysplastic and inflammatory disease states, as well as carcinomas, should lead to a better understanding of the role of the ras gene in the etiology and pathogenesis of these lesions. Thus, MAbs to oncogene products, differentiation antigens, and tumor associated antigens now add a new dimension to both the study of the etiology and pathogenesis of human carcinomas, as well as in the development of strategies toward management of these neoplasmas.

Proposed Course of Research:

We plan to continue our analyses of ras p21 expression at the single cell level in human breast and colon carcinomas using Formalin-fixed tissue sections of surgically removed tissues and the ABC immunoperoxidase method. We believe that a careful evaluation of which particular cell types are expressing ras p21 at specific stages of disease progression may lead to a better understanding of the role, if any, of ras p21 in the pathogenesis of these adenocarcinomas. We plan to continue our development and use of the liquid competition RIA for the quantitation of ras p21 in cells in culture as well as in tissues from animal models and humans. The use of purified recombinant ras p21 as a standard allows us to define absolute amounts of ras p21 per cell or per ug of protein. The use of both the RIAs and immunohistochemical assays for ras p21 should lead to a better understanding of the role of the ras gene in the etiology of human carcinoma.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09002-03 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chrecters or less. Title must fit on one line between the borders.)*

Transforming Growth Factors in Rodent Mammary Tumors and Transformed Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David S. Salomon	Supv. Res. Biologist	LTIB, DCBD, NCI
Atul Sahai	Visiting Fellow	LTIB, DCBD, NCI
William R. Kidwell	Chief, Cell Cycle Regulation Section	LTIB, DCBD, NCI
Robert H. Bassin	Chief, Biochem. of Oncogenes Section	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Robert Pardue, Triton Biosciences Inc., Houston, TX
James Tam, Rockefeller University, New York, NY

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.3	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A variety of viral and chemical transformed cells and spontaneously arising tumor cells produce a family of growth regulators collectively known as transforming growth factors (TGFs). TGFs are able to confer upon normal, nontransformed cells several properties associated with the transformed phenotype and may be involved in the autocrine growth of these cells. Some of these TGFs, namely alpha-TGF, are structurally related to epidermal growth factor (EGF). EGF is required for the growth and survival of normal rodent mammary epithelial cells in a serum-free, hormone-defined medium. However, in contrast, rat mammary tumor cells obtained from chemically-induced rat mammary adenocarcinomas exhibit a diminished response to EGF. This reduced responsiveness to EGF is due to the production of an alpha-TGF-like growth factor, mammary tumor factor (MTF), by these tumor cells. MTF is a heat labile protein whose activity is destroyed by reduction. MTF exists as two species with molecular weights of 68,000 and 6,000 and a pI of 5.2. MTF is mitogenic for normal mammary epithelial cells but not for the tumor cells from which it was derived. Since chemically-induced rat mammary tumors have either an elevated expression or an alteration in structure of the oncogene protein, $p21^{ras}$, we examined the potential role of TGFs in the transformation of NIH/3T3 cells produced by Kirsten murine sarcoma virus (v-Ki-ras onc gene) and in two cellular revertants (C11 and F2) derived from v-Ki-ras transformed 3T3 cells (DT). The revertant cell lines possess elevated levels of $p21^{ras}$ like DT cells yet fail to grow in soft agar or are tumorigenic in nude mice. However, like DT cells, the revertants lack detectable EGF receptors and produce TGFs. Unlike DT cells, the revertants fail to grow in soft agar even in the presence of exogenously supplied TGF. These studies suggest that the lesion(s) in the revertants are distal to the elevated expression of p21ras and production of TGFs and that the elevated production of TGFs is necessary, but may not be entirely sufficient for maintaining the transformed phenotype in ras transformed cells.

Project Description

Objectives:

To determine whether transforming growth factors (TGFs) are produced by rodent mammary tumors; to isolate and characterize these TGFs and to define the role of epidermal growth factor (EGF) and TGFs in rodent mammary epithelial cell proliferation and transformation. In addition, to elucidate the role of TGFs in transformation induced by the ras family of oncogenes and the molecular and biological interactions which may occur between TGFs and elevated or activated $p21^{ras}$, the oncogene protein of the ras gene family.

Methods Employed:

Primary cultures of normal rat and mouse mammary epithelial cells are prepared from ductal and alveolar organoids following a limited collagenase digestion and separation of the epithelial organoids from contaminating stromal fibroblasts on Ficoll gradients. Cultures of normal or tumor organoid epithelial cells are propagated in a serum-free, hormone-defined medium to facilitate the identification of novel mammary epithelial cell growth factors and to delineate the mechanism(s) by which these growth factors regulate mammary epithelial cell proliferation and basement membrane production. Primary cultures are maintained on tissue culture dishes coated with various species of collagen to examine the growth response of mammary epithelial cells to different mitogens as a function of the composition of the substrates and to determine the influence of the substrata composition on endogenous type IV collagen and laminin production. TGFs are isolated from the acid-treated, CM concentrates obtained from primary cultures of rat mammary tumor cells or Kirsten murine sarcoma virus transformed NIH/3T3 cells (DT) or two flat cellular revertants (C11 and F2) obtained from the DT cells by ouabain selection. TGFs are also isolated directly from chemically-induced [Dimethylbenze(alpha) anthracene (DMBA) and Nitrosomethylurea (NMU)] rat mammary adenocarcinomas by acid-ethanol extraction. TGFs are purified by gel filtration chromatography ion-exchange chromatography and isoelectric focusing (IEF). TGF activity is assayed by the ability to: (1) inhibit the binding of EGF to its receptor in a radioreceptor assay (RRA); (2) induce the anchorage-independent growth (AIG) of normal rat kidney (NRK) and 3T3 cells in soft agar and (3) stimulate the proliferation of normal rat mammary epithelial (RME), NRK and 3T3 cells in monolayer in serum-free medium.

Major Findings:

A. Role of EGF and TGFs in Rodent Mammary Epithelial Cell Proliferation.

Synthesis of an intact basement membrane is a prerequisite for the proliferation and survival of rodent mammary epithelial cells in vitro and in vivo. These results were gleamed from studies utilizing cis-hydroxyproline (CHP) which selectively blocks the synthesis of collagen and promotes the death of mammary epithelial cells. In vitro mammary epithelial cells can be rescued from the toxic effects of CHP by plating the cells on type IV

collagen, the collagen species which these cells normally synthesize as part of a basement membrane, but not on type I collagen or plastic. Furthermore, in serum-containing medium or serum-free medium containing EGF, insulin, transferrin, dexamethasone (dex), ascorbic acid and fetuin mammary epithelial cells exhibit a preferential attachment to and spreading on type IV collagen as compared to type I collagen or plastic. Cell proliferation in response to specific growth factors or hormones is also dependent upon the composition of the substratum. On plastic or type I collagen in serum-free medium mammary epithelial cells mitogenially respond to EGF and dex. However, on type IV collagen, the cells no longer require EGF and dex for growth. The refractoriness of the cells to EGF and dex on type IV substrata (i.e., plastic and type I collagen) suggested that these two mitogens may be enhancing the endogenous production of type IV collagen on plastic or type I collagen. It was found that EGF produced a 2 to 3 fold differential increase in the amount of type IV collagen on type I collagen or plastic without affecting collagen turnover while dex was found to suppress type IV collagen turnover apparently by inhibiting the production of a type IV specific collagenase. This differential responsiveness to EGF and glucocorticoids could not be accounted for by any change in either the number or affinity of cell surface or soluble receptors for EGF and glucocorticoids, respectively. Moreover, the response to EGF on type IV collagen production could be attenuated when the mammary epithelial cells were grown on an exogenous type IV collagen substratum. A variety of other hormones and growth factors which are mitogenic for mammary epithelial cells were subsequently found to differentially stimulate type IV collagen production in normal rat mammary epithelial cells and was correlated with the ability of these agents to promote mammary epithelial cell proliferation. These studies were then extended to the growth of neoplastic rat mammary epithelial cells. In contrast to normal mammary epithelial cells, mammary epithelial cells obtained from well differentiated, primary DMBA-induced rat mammary adenocarcinomas were found to be mitogenically less responsive to EGF. Furthermore, EGF was almost completely inactive in being able to stimulate type IV collagen synthesis in these tumor cells. These observations, coupled with the finding that DMBA mammary tumor cells possessed fewer EGF receptors than normal rat mammary epithelial cells (2.5×10^4 versus 8.4×10^4 receptor sites per cell, respectively) suggested that the transformed mammary cells might be elaborating an EGF-like TGF (alpha-TGF). Concentrated acid-treated CM obtained from primary cultures of DMBA rat mammary tumor cells or the acid-ethanol extracts prepared directly from the DMBA tumors were therefore screened for the presence of alpha-TGF activities. Alpha-TGF activity, mammary tumor factor (MTG), was found in the CM concentrates and in the crude acid-ethanol tumor extracts. These mammary-associated TGFS were found to be acid stable but head and dithiothreitol sensitive proteins. The TGFs could inhibit the binding of EGF, but not insulin, to mouse 3T3 or mouse embryonal carcinoma cells in a RRA. In addition, the mammary tumor-associated TGFs could induce the AIG of NRK and 3T3 cells in soft agar and could stimulate the anchorage-dependent growth in monolayer culture of NRK, 3T3, chick embryo fibroblasts and normal rat mammary epithelial cells. Finally, they are able to produce a 2 to 10-fold differential increase in collagen production in NRK and Balbc/3T3 cells (type I collagen) or in normal rat mammary epithelial cells (type IV

collagen). However, these activities fail to stimulate the proliferation of or collagen production in primary cultures of rat mammary adenocarcinomas. In contrast to primary DMBA and NMU tumors, little or no TGF activity could be detected in transplantable tumors derived from the primary DMBA or NMU tumors. Since the primary tumors are well differentiated adenocarcinomas, while their transplantable counterparts are relatively undifferentiated carcinomas, it is possible that the presence of elevated levels of TGFs may be indicative of the degree of cellular differentiation in these tumors as influenced by various mammogenic hormones.

B. Relationship of Retrovirus ras Gene Expression and TGF Production in Kirsten Murine Sarcoma Virus Transformed Cells.

There is recent evidence to suggest that the elevated expression or mutational activation of the ras family of oncogenes may be involved in either the etiology and/or progression of rodent mammary adenocarcinomas induced by chemical carcinogens such as DMBA and NMU. To determine whether there may be some functional significance between ras activation and the production of TGFs, we have utilized a well defined model system established by one of us (R.B.). Kirsten murine sarcoma virus transformed mouse NIH/3T3 cells (DT cells) provide a useful in vitro model system in which to study the production of TGFs and to relate the levels of TGF to the expression of the ras gene product, $p21^{ras}$. Cellular revertants of the transformed DT cells have been isolated (C11 and F2). These elevated levels of $p21^{ras}$ like the DT cells yet are no longer transformed since they fail to exhibit AIG in soft agar or form tumors in nude mice. It was found that these revertants, like the parental transformed DT cells, lack any detectable EGF receptors on their cell surface. Concentrated CM from the DT and revertant cells was able to: (1) inhibit the binding of EGF to normal NIH/3T3 cells in a RRA; and, (2) stimulate the AIG of NRK, NIH/3T3 and NR6/3T3 (an EGF receptorless variant of 3T3 cells) cells in soft agar. More importantly, although the C11 and F2 revertants produce both alpha and beta-TGFs, these same cells are unable to respond to these same activities in that they fail to grow in agar even in the presence of these TGFs derived from their CM or the CM of the DT cells. These results suggest that the cellular genomic lesions(s) in the revertant cells must be distal to both the elevated expression of $p21^{ras}$ and the elevated production of TGFs with respect to these cells not being transformed. These results also suggest that the elevated production of TGFs may be necessary, but may not be entirely sufficient to maintain the transformed phenotype in ras transformed cells.

Significance to Biomedical Research and the Program of the Institute:

The detection, characterization and purification of TGFs from rodent mammary adenocarcinomas should prove efficacious as a first step in obtaining sufficient amounts of these growth factors for generating immunological reagents. The generation of antibodies against these or related activities will aid in addressing whether normal rodent mammary epithelial cells possess comparable growth factors, are synthesizing TGFs and, if so, whether the synthesis of these activities can be modulated

by mammotropic or lactogenic hormones. Moreover, elevated levels of these TGFs may be indicative of preneoplastic mammary epithelial cells and the presence of more differentiated tumor cells. Furthermore, elevated levels or activated $p21^{ras}$ may be involved in regulating the production of these mammary-derived TGFs.

Proposed Course of Research:

Experiments are in progress to further characterize and purify these rodent mammary TGFs; to generate either monoclonal or polyclonal antibodies against these activities; to study their synthesis and turnover in normal and tumor rat mammary epithelial cells with respect to the ability of other mammotropic hormones to modulate their production; to determine whether the elevated production of TGFs is unique to ras transformed cells or is a more general consequence of transformation by other oncogenes and to determine the molecular and biological interactions between $p21^{ras}$ and TGFs in rat mammary epithelial cells and mouse NIH/3T3 fibroblasts.

Publications:

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Bano, M., Zwiebel, J.A., Salomon, D.S. and Kidwell, W.R.: Detection and partial characterization of collagen synthesis stimulating activities in rat mammary adenocarcinomas. J. Biol. Chem. 258: 2729-2735, 1983.

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Anderson, W.B. and Salomon, D.S.: Calcium, phospholipid-dependent protein kinase C as a cellular receptor for phorbol ester tumor promotors: possible role in modulating cell growth and tumor promotin. In J.F. Kuo (Ed.): CRC in Biochemistry-Phospholipids and Cellular Regulation CRC Press Inc., Boca Raton, Florida. (In press).

Panneerselvam, M., Bano, M., Kidwell, W.R., and Salomon, D.S.: Stimulation of type I collagen fibronectin and laminin synthesis by epidermal growth factor (EGF) and 12-O-tetradecanoylphorbol-13-acetate (TPA). Biochem. et Biophys. Acta. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09003-03 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transforming Growth Factors from Human Mammary Tissues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David S. Salomon	Supv. Res. Biologist	LTIB, DCBD, NCI
Isabelle Perroteau	Visiting Fellow	LTIB, DCBD, NCI
William R. Kidwell	Chief, Cell Cycle Regulation Section	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Robert Pardue, Triton Biosciences, Inc., Houston, TX
James Tam, Rockefeller University, New York, NY

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	1.7	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The presence and role of transforming growth factors (TGFs) in human mammary carcinoma cells is being defined. Alpha-TGFs resemble epidermal growth factor (EGF) in that they are able to compete with EGF mouse and human EGF but fail to cross-react with antibodies generated against these two species of EGF. Alpha-TGFs can be recovered from the acid-treated, concentrated conditioned medium (CM) of a human mammary carcinoma cell line, MCF-7, and ten individual clones derived from this cell line. The level of TGF activity associated with the CM varied amongst the different MCF-7 clones and showed no correlation with either the number of EGF receptors expressed on these cells or with the intrinsic ability of these clones to grow in soft agar as colonies. The TGF in the CM is an acid heat stable peptide whose activity is destroyed by reduction. It has a molecular weight of approximately 6000 and a pI of 4.0 TGF activity can also be detected in the crude, acid-ethanol extracts prepared from MCF-7 cells propagated as tumors in nude mice, in the acid-ethanol extracts obtained from two transplantable human mammary adenocarcinomas, Clouser I and II, and in 3 out of 4 primary human breast carcinoma samples. Relatively high levels of TGF activity can be found in crude, delipidated, decaseinated human milk obtained from at least 30 individual donors. The levels of TGF in the milk samples vary from individual donors and are generally highest in clostrum. Following isoelectric focusing (IEF), three distinct TGF species can be detected in the human breast tumor samples with identical species found in human milk. The TGF with a pI of 3.8 - 4.0, mammary-derived growth factor-II (MDGF-II), has been purified approximately 10,000-fold. This alpha-TGF species is virtually identical to the species found in the CM from MCF-7 cells. This acidic alpha-TGF is biologically and physiochemically distinct from the major species of human EGF in milk.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To determine whether transforming growth factors (TGFs) are produced by human breast carcinoma cells; to isolate and characterize these human TGFs and to determine whether their presence is correlated with other phenotypic properties such as growth rate, anchorage-independent growth, tumorigenicity, estrogen and progesterone receptor status and expression of specific tumor-associated cell surface antigens.

Methods Employed:

MCF-7 cells were obtained from the Breast Cancer Task Force. The MCF-7 clones were generally supplied by Dr. P. Hand, LTIB. MCF-7 cells were propagated as tumors in nude female Balbc/mice (nu^+/nu^+) supplemented with estrogen pellets. Clouser I and II transplantable mammary adenocarcinomas were isolated from a biopsy and maintained in nude mice. Biopsies of human breast tumor samples were obtained from Dr. Patrick Byrne, Georgetown University Hospital. Human milk was obtained from the Georgetown University Hospital Human Milk Bank from individual donors (30-50 ml expressed/donor). TGFs were isolated from the acid-treated, concentrated conditioned medium (CM) of MCF-7 cells, from the acid-ethanol extracts prepared from xenografts in nude mice or primary biopsies, and from crude, delipidated, decaseinated pooled human milk samples. TGFs were physiochemically characterized by isoelectric focusing (IEF), gel filtration high performance liquid chromatography (HPLC) and reverse-phase HPLC. TGF activity was monitored by the ability of aliquots to: (1) inhibit the binding of ^{125}I-EGF to specific EGF receptors on mouse NIH/3T3 cells or to isolate membranes obtained from human A431 epidermoid carcinoma cells; and (2) induce the anchorage-independent growth (AIG) of normal rat kidney (NRK) and mouse Mm5mt/c_1 C3H mammary tumor cells in soft agar.

Major Findings:

We have previously demonstrated that an alpha-TGF can be recovered from the concentrated CM and crude acid-ethanol extracts of rat mammary tumor cells. The present project was, therefore, undertaken to determine whether a comparable activity(ies) could be detected in human mammary tumor cells. MCF-7 human mammary carcinoma cells (established from a pleural effusion) were grown in serum-free FEIT medium in the absence of EGF. Concentrated CM from the MCF-7 cells and ten individual clones derived from this cell line contain TGF activity. The major species of TGF associated with the CM has a molecular weight of approximately 6,000 and a pI of 4.0. The biological activity associated with this species is heat and acid stable but destroyed by reduction. No correlations were observed between the number of detectable EGF receptors on the individual MCF-7 clones (range 3 to 6 x 10^3 receptor sites/cell); and the amount of alpha-TGF activity which could be detected in the CM from those clones which varied in potency with respect to inducing the AIG of NRK cells in soft agar and the inhibition of binding of EGF to NIH/3T3 cells.

Furthermore, the amount of TGFs associated with the CM from the various clones showed no correlation with the intrinsic ability of these cells to grow in soft agar. These results suggest that the original MCF-7 parental cell line contains a heterogeneous population of cells, some of which may be relatively high or low producers of both alpha and beta-TGFs. These TGFs are apparently not restricted to the CM. Comparable levels of TGFs were found in the crude, acid-ethanol extracts prepared from MCF-7 cells maintained in nude mice as tumors, in the crude extracts prepared from two transplantable human mammary adenocarcinomas, Clouse I and II and in 3 out of 4 primary human breast tumor samples. Further purification of these human mammary tumor-associated TGFs required a rich source for these activities since it became apparent that the human mammary tumors or the CM from MCF-7 cells were a relatively poor source for obtaining sufficient quantities of these activities and since the overall recovery of these activities from those starting materials was generally low. We discovered that crude human milk contains a considerable amount of TGF activity. The TGF activity in different milk preparations varied among individual donors and was found to be highest in human clostrum. Since human milk also contains EGF (approximately 50 ng/ml) and since EGF has been functionally classified as an alpha-TGF, it was necessary to more fully characterize these milk-associated TGFs. Following IEF, the TGF activity in crude delipidated, decaseinated human milk could be resolved into three major species with pI's of 3.8-4.0, 6.0-6.2 and 6.8-7.0. Comparable isoelectric variants were also observed in the human breast tumor extracts after IEF. The TGF with a pI of 4.0 from human milk which we have operationally designated as mammary-derived growth factor II (MDGF-II) has been purified approximately 10,000-fold and has been found to be virtually identical to the TGF which is associated with the CM from the MCF-7 cells. MDGF-II differs from the major species of human EGF (pI 4.5) in its biological and physiochemical properties. Furthermore, little or no human EGF could be detected in this preparation or in the other more neutral TGF fractions by RIA, using a rabbit polyclonal anti-human EGF antiserum. MDGF-II is capable of stimulating the AIG of NRK, mouse mammary tumor, and MCF-7 cells in soft agar. The biological activity of MDGF-II is completely blocked by a mouse anti-EGF receptor monoclonal antibody suggesting that MDGF-II is an alpha-TGF whose activity requires interaction through the EGF receptor.

Significance to Biomedical Research and the Program of the Institute:

The isolation, characterization, and purification of TGFs from human mammary tumors should prove useful in the possible diagnostic detection and pathological staging of human breast tumors assuming that: (1) increased production of TGFs is associated with neoplastic or preneoplastic cells; and (2) that more differentiated tumors (i.e., adenocarcinomas) express relatively higher levels of TGFs than their more undifferentiated counterparts). The acquisition of immunological reagents against these purified mammary-associated TGFs will aid in addressing these questions and whether normal human mammary epithelial cells have the capacity to synthesize and secrete these growth factors in response to different hormones. Moreover, the ability to quantify the levels of $p21^{ras}$ using a recently developed RIA will facilitate studies to determine whether there is any correlation

between elevated levels of $p21^{ras}$ and the enhanced production of TGFs in these types of tumors.

Proposed Course of Research:

Studies are in progress to further purify sufficient amounts of these human mammary TGFs on a preparative level and to utilize these purified growth factors as immunogens to obtain monoclonal antibodies. Experiments are also underway to study the synthesis and turnover of these TGFs in normal and neoplastic mammary epithelial cells in response to various mammotropic hormones and to relate the production of these TGFs to the expression of $p21^{ras}$.

Publications:

Salomon, D.S., Zwiebel, J.A., Bano, M., Losonczy, I., Fehnel, P. and Kidwell, W.R.: Presence of transforming growth factors in human breast cancer cells. Cancer Res. 44: 4069-4077, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09009-04 LTIB

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October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chrecters or less. Title must fit on one line between the borders.)*

Augmentation of Tumor Antigen Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

John W. Greiner	Senior Staff Fellow	LTIB, DCBD, NCI
Patricia Horan Hand	Chemist	LTIB, DCBD, NCI
Martin Tobi	Visiting Associate	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. S. Pestka, Institute of Molecular Biology, Hoffman La Roche, Nutley, NJ;
Dr. P. Fisher, Columbia University, New York, New York;
Dr. P. Noguchi, Bureau of Biologics, FDA

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.4	2.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Within human breast and colon carcinoma lesions, as well as established human tumor cell lines, there exists extensive heterogeneity in the expression of defined tumor antigens as recognized by the binding of monoclonal antibodies (MAbs). This heterogeneity of tumor antigen expression can be found within defined areas of the tumor and within subcellular compartments of a single tumor cell. These studies have shown that human tumor cells have the ability to intrinsically regulate the expression of the tumor antigens. Factors such as 1) cell cycle kinetics, 2) long-term growth in vitro, 3) clonal variability, and 4) growth of tumor cells in three-dimensional structures all alter the antigen phenotype of human tumor cells. The apparent ability of human tumor cells to intrinsically modulate their tumor antigen expression provides insight into the factors contributing to the extensive antigenic heterogeneity observed in these cell populations. Studies were conducted to identify those biological response modifiers that can override increase in the cell surface binding of monoclonal antibodies. Recombinant human leukocyte interferon (IFN-alpha A) was found to increase the binding of specific MAbs to the surface of human breast and colon carcinoma cells in a dose-dependent manner. Utilizing 10-100 units IFN-alpha A, tumor antigen expression can be dramatically increased independent of any change in tumor cell proliferation. Cell sorter analysis revealed that the IFN-alpha A mediated increase in tumor antigen expression is a result of an increased amount of antigen per cell as well as the recruitment of previously antigen negative cells to antigen positive. These studies may thus lead to: (i) a better definition of the transformed phenotype and (ii) the development of new strategies to enhance the in vivo binding of monoclonal antibodies to tumor antigens.

GPO 914-916

Project Description

Objectives:

To use monoclonal antibodies to define the extent of antigenic heterogeneity for the expression of certain tumor antigens associated with human breast and colon carcinoma cells. To determine the extent of antigenic modulation that occurs both *in vitro* and *in vivo*. To describe those intrinsic factors that are capable of modulating antigen expression. To develop strategies using biological response modifiers to overcome the intrinsic antigen modulation so as to selectively enhance the surface expression of specific tumor antigens.

Methods Employed:

Antigenic heterogeneity was defined by the differential binding of purified monoclonal antibodies to fixed tissue sections from primary as well as metastatic carcinoma lesions and from cytospin preparations of human tumor cells grown *in vitro* using the immunoperoxidase technique. The binding of the monoclonal antibody to the surface of human breast and colon tumor cells was measured by a radioimmunoassay using live, intact cells and by fluorescence activated cell sorting.

Major Findings:

I. Differential Expression of Defined Tumor Associated Antigens (TAA) Within and Among Carcinoma Masses.

Our studies have revealed a wide range of antigenic phenotypes among carcinoma masses. For example, ten different antigenic phenotypes emerged when 39 histologically similar infiltrating duct carcinomas of the breast were reacted with four MAbs that recognize four different tumor antigens. One observed pattern of antigenic heterogeneity was that one area within a tumor section was antigen-positive while another area in the same section was antigen-negative. A more common type of antigenic heterogeneity was observed among cells in a given area of a tumor mass. This type of antigenic diversity, termed "patchwork," reveals tumor cells expressing a specific TAA directly adjacent to tumor cells negative for the same antigen.

Furthermore, differential reactivity of the MAb at distinct subcellular compartments of the tumor cell at different sites within the tumor mass were also evident. For example, the TAG-72 antigen could be found as a cytoplasmic protein in one area of the tumor and as a cell surface membrane component in a different area within the same tumor mass. Similar degrees of heterogeneity of tumor antigen-expression were observed within established human tumor cell lines. The MCF-7 human mammary tumor cell line was tested for the presence of TAAs using cytospin/immunoperoxidase methodology. This MCF-7 cell line contains various subpopulations of cells as defined by variability in expression of TAAs reactive with several MAbs, i.e., antigen positive MCF-7 cells were seen adjacent to cells which scored negative.

II. Intrinsic Modulation of Tumor Antigen Expression by Human Tumor Cells.

(a) Relationship of growth kinetics to antigen expression. The cell surface binding of MAb B6.2 was monitored on MCF-7 mammary carcinoma cells during logarithmic growth and at density-dependent arrest of cell proliferation. Antibody binding was monitored each day for seven consecutive days after passage. One day after passage, B6.2 was strongly reactive with the MCF-7 cells. Over the following seven days, the reactivity of the monoclonal decreased progressively and was lowest at density dependent arrest. Passage of these cells on day 7 resulted in the reappearance of the antigen within 24 hours, suggesting the expression of the 90 kd tumor antigen is cell cycle dependent. Similar results were observed with monoclonal B38.1

To investigate the relationship of antigen expression to cell cycle phase, day 7 cells at density-dependent arrest were passed and monitored 24 hours later for both DNA content and cell surface antigen expression via fluorescent activated cell sorter analyses (FACS). DNA staining by Hoechst dye demonstrated that the majority of cells were in the G_0/G_1 phase. The remainder of cells were distributed evenly throughout S-phase and G_2/M phase. G_0/G_1 cells express background fluorescence. In contrast, MAb B6.2, as well as MAb B38.1 were most reactive with cells in S-phase.

(b) Modulation of tumor antigen expression following binding of a monoclonal antibody. In light of the phenomena of "capping," "internalization," and "shedding" of lymphocyte surface antigens after exposure to MAbs, studies were undertaken to determine the effect of prolonged exposure of MCF-7 mammary carcinoma cells to MAbs B6.2 and B38.1 on cell surface expression of the reactive antigens. MCF-7 cells were incubated in the presence of monoclonal B6.2 or B38.1 for two hours, washed extensively and then refed with growth medium. Cells were then incubated for an additional 22 hours and analyzed following the addition of G/M fluorescein isothyiocyanate. The fluorescence profile was again similar to that achieved during a 2 hour or 24 hour antibody incubation. The membrane expression of the reactive antigens therefore appears to be stable, despite continued exposure to antibody. These studies have important implications if MAbs to carcinoma associated antigens are to be coupled with toxins in protocols for cell killing in which the MAb-toxin complex must not only bind but be internalized by target cells.

(c) Antigenic drift of human breast tumor cell populations. Studies were conducted to determine if the antigenic phenotypes of human mammary tumor cell lines changed over an extended period of time. The BT-20 cell line, obtained at passage 288, was serially passaged and assayed at each passage level during S-phase of the growth cycle. Cell surface HLA-A, B, C antigen, detected by MAb W6/32, was present at all passage levels, as was the TAA detected by MAb B38.1. The 90 Kd antigen detected by MAb B6.2 was expressed on the BT-20 cell surface up to passage 319, but then disappeared. This phenomenon was observed in three separate experiments at approximately the same passage levels. In a related series of experiments, four MCF-7 mammary carcinoma cell lines were obtained from different sources and were examined for surface expression of several TAAs. MAb B139, which recognizes an antigen on the surface of all human cells reacted with all four cell lines.

However, when other MAbs were tested, several different antigenic phenotypes emerged. The MCF-7 (BCTF) contained all the tumor associated antigens assayed for, while the MCF-7 (GC) cell line expressed none. We believe this observation should serve as a cavaet to those investigators using established carcinoma cell lines attempting to correlate their results on antigenic phenotype with those in other laboratories.

(d) Clonal heterogeneity of tumor antigen expression within a human breast tumor cell line. To further characterize the antigenic heterogeneity of human mammary tumor cell populations, MCF-7 cells were cloned by end-point dilution. Ten different clones were obtained and assayed for expression of cell surface TAAs. The parent MCF-7 cells reacted most strongly with MAb B1.1 and least with MAb B72.3. Clone 6F1 had a parental antigenic phenotype. At least three additional major phenotypes were observed among the remaining clones. For example, clone 10B5 did not express the TAAs reactive with monoclonals B1.1, B6.2, and B72.3 although it did contain HLA-A, B, C and the normal human antigen detected by MAbs W6/32 and B139, respectively. A second phenotype, represented by clone 5H7, revealed a four-fold less expression of TAA as compared with the parent. Clone 5A9 showed weak binding for monoclonals B1.1 and B6.2 and a stronger reactivity with B72.3; this phenotype is the opposite of that seen with the parent cell line. Variation of expression of cell surface TAAs among the clones was corroborated using the immunoperoxidase/cytospin method. To determine the stability of the antigenic phenotype of the MCF-7 clones, each cloned cell line was monitored through a four-month period and assayed during log phase growth at approximately every other passage. While some of the MCF-7 clones maintained a stable antigenic phenotype, other clones underwent dramatic changes in their cell surface antigen expression. Clone 10B5 for example, did not express any of the three tumor antigens which react with MAbs B1.1, B6.2 and B72.3 for 6 passages. Subsequently, these antigens became increasingly expressed on the cell surface from passages 9 to 15. Such changes in antigenic phenotype appeared to be independent of the growth rate for the cloned cell lines.

(e) Effect of spacial configuration on tumor antigen expression. There exists a dichotomy in the expression of TAG-72 in carcinoma biopsy material vs. established carcinoma cell lines. While 44% (25/56) of human breast carcinoma and 80% (16/20) of colon carcinoma biopsies express TAG-72, only 1 of 25 breast cancer cell lines [MCF-7(one variant)] an 1 of 18 colon cancer cell lines (LS-174T) express this antigen. Furthermore, TAG-72 expression in these 2 cell lines was shown to be a property of only 1-5% cells. Attempts to enhance TAG-72 expression in LS-174T cells by propagation on extracellular matrix proteins such as collagen, laminin, fibronectin, or in serum-containing or serum free/hormone supplemented media all proved unsuccessful. A pronounced increase in TAG-72 expression was observed when the LS-174T cells were grown under culture conditions which promoted three-dimensional growth. For example, LS-174T cells grown in spheroid or suspension cultures had a 2- to 7-fold higher level of expression of the TAG-72 antigen, while those grown on agar plugs demonstrated a 10-fold increase. When the LS-174T cells were injected into athymic mice, the level of TAG-72 antigen in the tumors was 100-fold higher than that measured in the cells in the culture. Thus, it seems that spacial configuration, or the cell-to-cell communication

developed in three-dimensional growth, can facilitate the surface expression of a specific tumor associated antigen.

III. Use of Recombinant Human Interferon to Alter the Expression of Tumor Antigens in Human Breast and Colon Carcinoma Cells.

(a) Changes in tumor antigen expression following treatment with human leukocyte (Clone A) interferon (IFN-alpha A). Treatment of human breast or colon carcinoma cells with IFN-alpha A will increase the surface expression of specific TAAs. Utilizing MAbs B1.1, B6.2 and B72.3, the effects of IFN-alpha A on the expression of the respective antigens were investigated on several different cell types. Treatment of the human breast carcinoma cell, MCF-7, with 10-1,000 antiviral units of IFN-alpha A increased the surface expression of all three tumor antigens. For example, CEA expression rose 34%, 94% and 169%, respectively, after a 24 hour incubation in medium containing 10, 100, 1,000 units IFN-alpha A/ml. The human colon carcinoma cell, WiDr, expresses both CEA and the 90Kd B6.2-specific tumor antigen, but not the B72.3 reactive TAG-72 antigen. IFN-alpha A treatment increased the amount of B1.1 and B6.2 bound to the WiDr cell surface, but did not initiate TAG-72 expression. The expression of the 90Kd tumor antigen in the WiDr cell surface was increased 41%, 82% and 134% following treatment with 10, 100, or 1,000 units IFN-alpha A. For comparison, the expression of the class I major histocompatibility antigen, HLA-A, B, C, was also increased following IFN-alpha A treatment. However, a second normal surface antigen which binds MAb B139 and has a surface distribution similar to that of HLA was unchanged by interferon treatment. Higher concentrations of IFN-alpha A that were cytostatic or cytotoxic were also less effective in enhancing TAA expression. It should be noted that other types of human cells, both normal and neoplastic, that do not express these tumor antigens remain negative after interferon treatment. One may suggest that interferon administration may effectively convert a human tumor cell population to one that is more antigen positive while the surrounding normal tissues remain tumor antigen negative.

(b) Fluorescence activated cell sorter analysis of IFN-alpha A mediated increase in tumor antigen expression. The mechanism(s) by which IFN-alpha A increases the expression of tumor antigens on the surface of human tumor cells is unknown. Human colon carcinoma cells, WiDr, were treated with IFN-alpha A for 24 h and the level of MAb B1.1 reactivity to the surface of these cells was compared with that on the untreated cells. IFN-alpha A treatment caused no change in either the size of the WiDr cells or their cellular DNA content. However, following the 24 h treatment with 1000 antiviral units of IFN-alpha A, there was a substantial increase in the mean fluorescent intensity per cell as well as an increase in the number of WiDr cells expressing the tumor antigen. For example, the percentage of WiDr cells that express the B1.1-reactive antigen rose from approximately 84% to 98%. Therefore, it seems that IFN-alpha A treatment results in a recruitment of human tumor cells that did not express a detectable level of tumor antigen to become antigen positive. The overall result of the recruitment of new antigen positive cells is a human tumor cell population which is more homogeneous in its expression of a tumor associated antigen.

One of the considerations for the detection of in situ carcinoma lesions by labeled MAbs is reducing the amount of labeled monoclonal. The administration of low levels of the antibody should increase the "signal:noise" ratio and make the localization of small lesions more efficient. Therefore, we compared the amount of radiolabeled B72.3 needed to localize a reference amount of antibody to the surface of MCF-7 cells before and after interferon treatment. Untreated MCF-7 cells needed 94 ng of the labeled MAb to bind 1,000 cpm. MCF cells treated with 1-1,000 units IFN-alpha A required substantially less of the MAb B72.3 to localize the same amount of radioactivity at the cell surface. For example, a 24 hour incubation with 1,000 units of IFN-alpha A reduced the required amount of ^{125}I-B72.3 from 94 ng to approximately 4 ng (24-fold). Thus, after interferon treatment the amount of labeled antibody can be significantly reduced while maintaining the same signal at the cell surface.

Significance to Biomedical Research and the Program of the Institute:

One characteristic exhibited by most, if not all, human tumor cells is the presence of extensive cellular heterogeneity as defined by numerous biological parameters. Antigenic heterogeneity is a well documented phenomenon of human breast, colon and other carcinomas. The presence of heterogeneity in the expression of tumor antigens on the surface of human breast and colon carcinoma cells will undoubtedly be a limiting factor in the utilization of monoclonal antibodies for tumor detection and/or therapy. The studies described here present an insight into the intrinsic characteristics of a human tumor cell which make it capable of altering its antigenic phenotype. One can postulate that such factors also contribute to the observed antigenic heterogeneity in tissue sections from human carcinomas as well as in human carcinoma cell lines. Antigenic changes associated with growth kinetics, prolonged maintenance of cell lines in vitro and cell-to-cell communication may be relevant to manifestation of the transformed and/or malignant cell type. A further understanding of the intrinsic cellular and molecular signals that modulate tumor antigen expression may help predict or control the specific antigenic phenotype of a human tumor cell population. As stated previously, the heterogeneity in the expression of surface tumor antigens will limit the efficiency of labeled monoclonal antibodies used for tumor detection and/or therapy. Experimental evidence indicates that certain biological response modifiers, such as recombinant human interferon, can transmit an external stimulus which seemingly overrides the intrinsic cellular controls resulting in an increased expression of tumor antigens on the surface of human carcinoma cells. The end result is a human tumor mass which is more homogeneous for tumor antigen expression. This may, in turn, yield increased binding of labeled monoclonal antibodies and thus facilitate tumor detection and therapy.

Proposed Course of Research:

We plan to determine whether other recombinant species of leukocyte interferon, i.e., B, C, D, F, I, J, K, have equal or greater abilities to modulate tumor antigen expression than IFN-alpha A. In addition, hybrid forms of leukocyte interferon along with fibroblast and immune interferons will be tested for their effects on tumor antigen expression. A major emphasis will be focused on determining the in vivo effects of the recombinant

interferons. Using human tumor cell lines, or xenografts from breast and colon carcinoma patients, the various types and combinations of interferons will be tested for their ability to enhance human tumor antigen expression as determined by antigen content within the tumor and the radiolocalization of a labeled MAb.

Other compounds will also be evaluated for their effects on the antigenic phenotype of human tumor cells. Additional studies will determine whether interferons in combination with other agents may synergistically augment the binding of monoclonal antibodies to human tumor antigens.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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Z01 CB 09012-02 LTIB

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October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Monoclonal Antibodies to Detect Occult Carcinoma Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Ann Thor	Medical Staff Fellow	LTIB, DCBD, NCI
Noriaki Ohuchi	Visiting Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Drs. W. Johnston and C. Szpak, Dept. of Pathology, Duke Univ., Durham NC:
Dr. S. Martin, Laboratory of Pathology, NCI, NIH;
Dr. H. Wolfe, Department of Pathology, Tufts University, Medford, MA

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A monoclonal antibody (MAb), designated B72.3, has been generated using membrane enriched fractions of a metastatic human breast carcinoma as the immunogen. Previous studies have demonstrated that the reactive antigen, a novel high molecular weight glycoprotein complex, can be detected in Formalin-fixed, paraffin-embedded tissue sections of human breast and colon carcinomas, and not in a variety of normal adult human tissues. We have now determined that MAb B72.3 may be used as an adjunct for diagnosis of adenocarcinoma in cytologic preparations of human effusions. Using the avidin-biotin complex method of immunoperoxidase staining and Formalin-fixed, paraffin-embedded cell suspensions, MAb B72.3 detected adenocarcinoma cells in effusions from all of 21 patients with adenocarcinoma of the breast. No reactivity was demonstrated in any cell type in benign effusions from 24 patients without cancer, or 13 patients with prior or extant cancer in other body sites; moreover, B72.3 showed no reactivity to leukemic or lymphomatous effusions, or apparent mesothelial cells from malignant effusions. Monoclonal antibody B72.3 also detected adenocarcinoma cells in cytologic effusion specimens from 12/12 patients with adenocarcinoma of the lung and 16/16 patients with adenocarcinoma of the ovary. Thus, these data suggest that the immunocytological application of MAb B72.3 should now be considered as an adjunct in the discrimination of adenocarcinoma cells from reactive mesothelial cells in the cytologic diagnosis of malignant effusions and may be applicable in the detection of occult tumor cells in needle aspirates as well as other biologic fluids.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To utilize monoclonal antibodies and immunohistochemical and immunocytochemical techniques as an adjunct to standard pathologic examination to (a) detect occult tumor carcinoma in human effusions and needle aspirates, and (b) phenotype specific types of tumor cells for use in differential diagnoses.

Methods Employed:

The monoclonal antibodies were generated against membrane-enriched extracts of metastatic breast carcinoma using hybridoma technology. All cytopathologic specimens of effusion fluid were clinical specimens fixed in three ways. All three cytologic preparations were reviewed by several investigators before a diagnosis was made. A slight modification of the avidin-biotin-peroxidase complex method was used for immunohistochemical studies of the cell button preparations. Positive controls, as well as an isotype identical MAb negative control were included in each assay. All sections stained were examined by at least two experienced investigators.

Major Findings:

The correct diagnostic recognition of cancer cells in serous effusions by routine cytopathologic methods can be extremely difficult. Cancer cells may be difficult to differentiate from reactive mesothelial cells; if sparsely scattered throughout benign inflammatory cells they may even be missed. When neoplastic cells are identified, the histologic tumor type or organ of origin may be difficult to determine. These problems are encountered daily in patients with known malignancies who develop effusions of the pleural, pericardial or peritoneal cavities either as a manifestation of metastatic disease, infection, or metabolic abnormality. Among malignant effusions, adenocarcinoma is the most frequent type of malignancy identified. The majority of tumors which metastatasize and cause malignant effusions are from the lung, breast, and female reproductive organs. Of even greater importance are those effusions from patients in whom there is no previous history of underlying malignancy. The question of a malignant process is ever present, and the correct recognition of malignant cells is perhaps more significant in terms of treatment and prognosis. The patient group without prior or extant cancer constitutes approximately 10% of all malignant effusions studied. Of these, the majority of malignant effusions are manifestations of metastatic adenocarcinoma from the breast, ovary and lung. We have previously shown that MAb B72.3 is reactive with human mammary and colon carcinoma cells, and is non-reactive or very weakly reactive with a variety of normal adult tissues. The reactive antigen, TAG-72, is a high molecular weight glycoprotein which has been shown to be immunoreactive in Formalin-fixed paraffin-embedded tissue sections using ABC immunoperoxidase methods. Formalin-fixed, paraffin-embedded cytologic effusion specimens were thus examined with MAb B72.3 to determine if malignant cells could be differentiated from benign cells on the basis of their expression of the B72.3 reactive antigen. The patient specimens had

been previously evaluated using standard cytology techniques including cytospins, membrane filters, and cell pellets embedded in paraffin blocks to make the initial diagnosis. These cytology preparations were reviewed by two cytopathologist to reconfirm the initial diagnosis, i.e., malignant or benign. The purpose of these studies was to determine if MAb B72.3 could be used as an adjunct for the detection of adenocarcinoma cells in human serous effusions. A total of 132 separate cell blocks representing individual specimens of effusion fluid from 115 patients from several diagnostic categories and disease entities were studied. All cytologic specimens of effusion fluid had been previously obtained for clinical diagnostic purposes from the pleural (n=95), peritoneal (n=30), or pericardial (n=7) cavities. From a centrifuged aliquot of heparinized effusion fluid, the pelleted sediment was mixed with an equal volume of 10% buffered Formalin and allowed to fix for one hour. After recentrifugation the fixed cell button was encased in melted agar, paraffin-embedded, and 5 micrometer sections were mounted on gelatin coated slides.

Twenty-four specimens of confirmed benign pleural effusions from 24 non-cancer patients were first reacted with MAb B72.3. In all cases, mesothelial cells as well as all other cell types were negative. More importantly, in 17 specimens of confirmed benign effusions from 13 patients with prior or extant cancer in other body sites, no B72.3 reactivity was observed in any cell type. The most common source of malignant cells in pleural effusions in women is adenocarcinoma of the breast. Twenty-three Formalin-fixed paraffin-embedded cell pellets from pleural effusions of 21 confirmed cases of mammary adenocarcinoma were examined with MAb B72.3 using the ABC immunoperoxidase method. MAb B72.3 reacted with malignant cells in 22 of 23 specimens, and all 21 of 21 patients examined (Table 2). A MOPC-21 isotype identical control MAb was negative in all cases. A heterogeneity in MAb B72.3 staining of malignant cells was seen in most specimens, i.e., not all malignant cells stained. The percentage of malignant cells reactive with MAb B72.3 varied from 5% to 100% with the majority of cases demonstrating at least 50% of the malignant cells staining. This heterogeneity has previously been shown with MAb B72.3 and fixed tissue sections of both primary and metastatic breast carcinomas. In the majority of effusions examined, the B72.3 reactive antigen was expressed in the cytoplasm of carcinoma cells, with staining occasionally being restricted to the cell membrane. As best as could be determined by cytologic criteria, there was no staining of benign mesothelial cells by MAb B72.3 in all 23 specimens examined.

We have previously shown that MAb B72.3 does not react with tissue sections of melanomas, sarcomas, and lymphomas. As expected, therefore, MAb B72.3 was shown to be non-reactive with effusions specimens containing hematologic malignancies including various leukemias and lymphomas. Thus MAb B72.3 may prove useful in those cytology cases where the histologic tumor cell type is in question. The importance of the above observations is best illustrated by the case reports recently reported. In both cases problems of diagnostic interpretation for the cytopathologist were addressed and aided by the use of MAb B72.3, i.e., discriminating tumor cells with bland cytologic features from reactive mesothelial cells and identifying the rare groups of malignant cells in an extremely cellular sample containing primary inflammatory cells.

MAb B72.3 has been reacted with confirmed malignant effusion specimens containing

a spectrum of tumor cell types. Because, with the exception of lung, breast and ovarian carcinoma, development of effusions is a relatively rare event, only limited numbers of each tumor type have been evaluated. Carcinoma cells in seven of eight effusions obtained from patients with confirmed adenocarcinoma of the uterus or vagina scored positive for reactivity with MAb B72.3. Positive reactivity of carcinoma cells with MAb B72.3 was also seen in 22 of 27 effusions specimens obtained from 19 patients with confirmed lung carcinoma of several histologic cell types. It should be noted, however, that malignant cells in all 19 specimens examined from 12 patients with confirmed adenocarcinoma of the lung were positive for MAb B72.3 reactivity. Similarly, all 19 effusion specimens from 16 patients with confirmed ovarian adenocarcinoma contained adenocarcinoma cells expressing the antigen reactive with MAb B72.3. As described above for breast carcinoma, an antigenic heterogeneity in carcinoma cell populations was also observed in most positive specimens.

Macrophages were positive for MAb B72.3 reactivity in four of sixty-six effusion specimens containing MAb B72.3 antigen positive adenocarcinoma cells. However, in all of these cases there was no difficulty in differentiating adenocarcinoma cells from macrophages because of the distinct cytomorphological characteristics of the two cell types. The presence of antigen in macrophages is most likely due to antigen which is shed by the carcinoma cells and processed by the macrophages, or by macrophage phagocytosis of cellular debris. Two pieces of evidence support this hypothesis: (a) the B72.3 reactive antigen is sometimes secreted by carcinoma cells, and (b) previous immunohistochemical studies on tissue sections of breast and colon carcinomas have shown that, in some cases, macrophages (or tissue histiocytes) adjacent to reactive carcinomas contain B72.3 reactive antigen, while macrophages far removed from the carcinoma do not. In 41 benign effusions examined, macrophages were routinely negative for MAb B72.3 reactive antigen.

Significance to Biomedical Research and the Program of the Institute:

The cytopathologic diagnosis of cells in serous effusions may be complex in that reactive mesothelial cells may be difficult to differentiate from tumor cells; and cancerous cells when present may be sparse. Approximately 10% of all serous effusions are found to contain malignant cells, particularly epithelial neoplasms including adenocarcinomas arising in the breast, ovary, and lung. The studies undertaken here demonstrate that MAb B72.3 can distinguish carcinoma cells from benign cells including reactive mesothelial cells. Furthermore, the data suggest that MAb B72.3 recognizes antigenic determinants unique for adenocarcinoma versus other malignant neoplasms. Thus, MAb B72.3 can be a useful adjunct to routine cytopathologic examination of serous effusions.

Proposed Course of Research:

Currently our efforts are focused on several areas: (1) the examination of more malignant and benign effusion, especially "borderline" cases in which the diagnosis of adenocarcinoma is not absolute, (2) adaptation of the cell button immunoperoxidase techniques for use with cytospin preparations, (3) adoption of the methods employed to serve as an adjunct for the detection of occult tumor cells in needle aspirate preparations.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05233-04 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Purification of and Radioimmunoassays for Human Carcinoma Associated Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David Colcher	Supv. Microbiologist	LTIB, DCBD, NCI
Virginia Johnson	Visiting Fellow	LTIB, DCBD, NCI
Peter Fuhrer	Expert	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

J. Magnani, Laboratory of Biochemical Pharmacology, NIADDK, NIH; H. Sears, Fox Chase Cancer Center, Philadelphia, PA.

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A competitive radioimmunoassay (RIA) for a tumor associated antigen (termed TAG-72) identified by monoclonal antibody B72.3 has been established. The distribution of TAG-72 in human tissues has been shown to be highly specific for carcinomas with no significant reactivity to normal tissues. The RIA was used to examine sera from patients with colorectal carcinomas, other malignancies and normal sera. A mean of 2.2 units/ml of TAG-72 was found in the normal sera. When a cut-off level of 3 standard deviations above the mean level of TAG-72 found in normals is used, no patient with inflammatory disease or other benign colon diseases exhibited abnormal levels of TAG-72. Thirty-five percent of sera from advanced colon cancer patients and patients with other carcinomas were positive for TAG-72. No increase in TAG-72 reactivity was seen in normal sera or that from patients with melanomas or sarcomas. Comparison of the TAG-72 levels in sera with antigens recognized by the monoclonal antibodies currently used to screen sera of carcinoma patients clearly demonstrated that TAG-72 is different from the other antigens, and that TAG-72 can be found in some sera where no antigen is detected by the commercially available MAb RIAs.

TAG-72 has been purified from extracts of a human colon carcinoma xenograft in athymic mice using molecular sieving and antibody affinity chromatography. It has an apparent molecular weight of $\geq 10^6$ daltons, and has been characterized as a mucin.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To identify and characterize tumor associated antigens (TAAs) detected by monoclonal antibodies that bind human carcinomas. To determine if these monoclonal antibodies bind to antigens distinct from known TAAs. To preparatively purify these TAAs to aid in the characterization of these antigens. To develop radioimmunoassays for their detection in human tissues and biological fluids. To determine: (1) if these TAAs are present preferentially in the serum of patients with carcinomas versus healthy donors and patients with other malignancies, (2) if the TAA levels in sera of patients with carcinomas correlate with tumor burden, (3) if changes in the TAA levels in serum correlate with clinical response to therapy, and (4) if appearance of the TAAs in serum is predictive of recurrence of a previous carcinoma.

Methods Employed:

The presence of TAAs, reactive with monoclonal antibodies in primary tumors, metastases and cell lines was determined by solid phase radioimmunoassays. The molecular weight of the antigens was determined by SDS polyacrylamide electrophoresis and Western blotting techniques. Purification of the antigens was performed by molecular sieving and antibody affinity column chromatography. A competitive radioimmunoassay was established to determine whether the antigens recognized by the monoclonal antibodies are present preferentially in the serum of carcinoma patients.

Major Findings:

(a) Radioimmunoassays for TAG-72. We set out to develop a competitive radioimmunoassay using a solid phase matrix to enable the screening of large numbers of samples easily. It was therefore necessary to optimize various aspects of the RIA including: the binding of MAb B72.3 to polyvinylchloride microtiter plates coated with TAG-72 positive cell extracts, the amount of antigen needed to coat the plate, the buffers used for coupling and the time and temperature of incubation for the antibody-antigen reaction. Antibody binding was maximized when approximately 5 ug of extract in PBS was dried into each well.

Since the sensitivity of competition RIAs is dependent on the levels of primary antibody used, assays were conducted to determine whether using radiolabeled B72.3 antibody or using unlabeled B72.3 followed by a radiolabeled second antibody would result in higher levels of counts bound to extracts with minimal inputs of antibody. More radioactivity was bound to extracts using the second antibody method; this was probably due to multiple binding sites for the ^{125}I-goat anti-mouse IgG antibodies to the mouse B72.3 IgG molecule. A series of standard curves were then constructed for the competition RIA using tumor cell extracts containing TAG-72 as competitor and MAb B72.3 at different concentrations. The sensitivity of the assay improved with limiting amounts of antibody as was manifested by a displacement in the standard curve. Subsequent assays were carried out using a MAb B72.3 concentration of 10 ng/ml (0.5 ng/well) for each sample using the radiolabeled second antibody method.

The incubation time and temperature of the antibody-antigen reaction were varied to optimize both the solid phase RIA and the liquid phase competition RIA. We observed that overnight incubations at 4°C for the primary and secondary antibodies yielded maximal binding, while the antigen-antibody reaction in the liquid phase was essentially complete after a one hour incubation at either 37°C or 4°C.

Before using the competition RIA to examine serum from patients with carcinomas it was necessary to consider the effects, if any, of normal human serum on the competition RIA. This was first examined using pooled normal human sera. After normalizing binding curves with and without serum for 100% binding of the antibody to the plate in the absence of competitor, the competition curves for the TAG-72 diluted in assay buffer with and without serum were in close agreement. This validated the use of this competitive RIA for the screening of sera.

(b) Assay of Human Serum for TAG-72. Sera from 16 patients with advanced colorectal cancer and from 12 apparently normal donors were assayed for TAG-72 using the competitive RIA. The presence of TAG-72 antigen in sera resulting in a decrease in the amount of MAb B72.3 available for binding to the plate, is clearly seen for several of the serum samples from patients with colon carcinomas. All normal sera as well as the sera from several of the carcinoma patients showed little or no competition.

The distribution of TAG-72 was then examined in a larger sampling of the sera from patients with colorectal carcinomas and other malignancies. A mean of 2.2 units/ml of TAG-72 was found in the normal sera. Some patients with advanced colon carcinomas had TAG-72 levels as much as 33 times the mean of the normals. Sera from the pre-operative colon cancer patients and from patients with an early recurrence had TAG-72 levels above the mean for the normals, but few sera had levels of greater than 3 standard deviations above the mean of the normals. None of the sera from patients with inflammatory or benign diseases had significant TAG-72 levels in their sera; their mean values were similar to that of the normals. When a cut-off level of 3 standard deviations above the mean level of TAG-72 found in normals is used, no patient with inflammatory disease (0/10) or other benign colon diseases (0/9) exhibited abnormal levels of TAG-72. A patient with local infiltrating disease and one patient classified as "early recurrence" had increased levels of TAG-72. Seven out of 20 (35%) sera from advanced colon cancer patients and sera from 7 out of 20 patients with other carcinomas were positive for TAG-72. No increase in TAG-72 reactivity was seen in normal sera or that from patients with melanomas or sarcomas.

(c) Comparison of TAG-72 to Other Tumor Antigens. The presence of elevated levels of TAG-72 antigen in human sera was compared to serum levels of other tumor antigens detected by MAbs as measured with commercial RIA kits. The occurrence of carcinoma-associated antigens in selected sera from colon carcinoma patients was examined. The results demonstrate that some patient sera that scored negative in all the CEA, CA 19-9, and CA125 assays had increased levels of TAG-72. The inverse was also seen in two patients whose sera were both low in TAG-72 antigen and high in CEA and GICA antigens. These results clearly demonstrate that TAG-72 is different from the antigens recognized by the monoclonal

antibodies currently used to screen sera of carcinoma patients, and that TAG-72 can be found in some sera where no antigen is detected by the commercially available MAb RIAs.

(d) Identification of a Source for TAG-72. Monoclonal antibody B72.3 was generated by immunizing mice with extracts of a human breast tumor metastasis to the liver. Although this tumor has proven to be a rich source of TAG-72, its limited availability necessitated the identification of an alternate source of TAG-72 for purification studies. B72.3 has been shown to bind to over 50% of breast carcinomas and over 80% of colon carcinomas using immunoperoxidase techniques. In contrast, this monoclonal antibody exhibits only weak binding to 1/28 mammary tumor cell lines (MCF-7) and 1/18 colon carcinoma cell lines (LS-174T). The discrepancy between the abundance of TAG-72 in tumors and its relative absence from cell cultures led to studies of the presence of TAG-72 in tumor xenografts in athymic mice established from a number of human carcinoma cell lines and transplantable tumors. Protein extracts were made from a number of breast and colon carcinoma xenografts grown in athymic mice and assayed for TAG-72 content. The tumors established from the LS-174T human colon carcinoma cell line had levels of TAG-72 similar to those found in the human breast tumor metastasis as measured in the competitive RIA described above. Studies were then undertaken to determine if the antigen found in LS-174T xenografts had a similar size distribution as that found in human primary and metastatic colon and breast carcinomas. Protein extracts were prepared from human colon and breast carcinomas as well as LS-174T xenografts and analyzed using Western blotting methods. The antigen detected by MAb B72.3 in all the antigen positive tumors exhibited a broad size distribution of approximately 200,000 to greater than 10^6 daltons. Large molecular weight molecules, especially mucins, are subject to shearing, resulting in the breakage of a high molecular weight form into lower molecular weight fragments. Since the extraction protocol for the antigen consisted of nitrogen cavitation followed by sonication, the range of sizes that are observed may be due to fragmentation of a high molecular weight molecule. To test this hypothesis more gentle extraction techniques were used. The resulting extracts were then run on 3-10% polyacrylamide gels and analyzed using Western blots. A higher molecular weight molecule ($\geq 10^6$) was apparent, with an absence of lower Mr forms. This indicates that the low molecular weight molecules are most likely the result of the sonication step used in the original extraction protocol. These studies have shown that the LS-174T xenograft, therefore, is an excellent source of TAG-72 and the antigen from this tumor has a molecular weight range similar to that observed in patient material. The LS-174T xenografts were therefore used in subsequent studies for the purification and characterization of TAG-72.

(e) Purification of TAG-72. LS-174T colon carcinoma xenografts were homogenized and centrifuged to remove nuclei (1000 Xg) and microsomes (10,000 Xg). The post-microsomal supernatant was applied to a Sepharose CL-4B column to separate low molecular weight proteins from TAG-72. Fractions were collected and analyzed for protein and TAG-72 levels. The TAG-72 was found in the column void, as expected with an antigen of this size, while the bulk of the protein was effectively separated by the gel filtration column. The fractions containing the TAG-72 antigen were pooled and loaded onto an affinity column using the HW-65F matrix coupled with 180 mg of B72.3 IgG. Although the majority of the

protein flowed through the column, most of the TAG-72 bound to the affinity matrix and was subsequently eluted using 3M NaI. Extensive studies have shown that the TAG-72 antigen can be more effectively eluted from the affinity matrix with high salt without significant loss of immunoreactivity as compared to using low pH. The eluted TAG-72 peak was then pooled, dialyzed against Tris-buffered saline, concentrated and then rechromatographed on a smaller column containing the same affinity matrix to obtain an increased degree of purification.

The purification was monitored using a competitive radioimmunoassay to quantitate TAG-72 activity, and a Lowry assay to quantitate the protein yield. The purification was also monitored by SDS-polyacrylamide gel electrophoresis to determine the purity of the TAG-72 antigen. The Coomassie stained gels demonstrate a loss in low molecular weight proteins as a function of the purification process, while the amount of high molecular weight PAS staining material increases with the purification. The PAS staining material does not stain with Coomassie Blue; this is indicative that this material is heavily glycosylated. The size and distribution of the TAG-72 does not appear to change with purification indicating that there is no additional shearing due to the purification methods used. We were able to purify the TAG-72 antigen at least 45-fold with a recovery of approximately 14%. These values are underestimates because they are based on a comparison of the final yield of TAG-72 with the amount of TAG-72 in the post-microsomal supernatant rather than the total extract. Further studies are in progress to more accurately determine the homogeneity of the purified TAG-72 and the recovery of TAG-72 throughout the various chromatography procedures.

Significance to Biomedical Research and the Program of the Institute:

The identification and characterization of these TAAs will enable us to compare these antigens with TAAs from other sources. The purification of these TAAs will also aid in the establishment of radioimmunoassays for their detection in human tissues and/or biological fluids. These assays may prove useful in the diagnosis and prognosis of patients with carcinomas, in the detection of recurrence of the disease, and in monitoring the efficacy of various therapeutic regimens. Peptide and carbohydrate analysis of the purified antigens and comparison with other proteins may aid in the determination of the biological activity and significance of the TAAs. The amino acid sequence of the TAAs will (a) enable the synthesis of portions of the peptide that may be useful in subsequent radioimmunoassays and (b) enable the synthesis of a cDNA copy that will be useful in studies to clone the genes coding for the TAAs.

Proposed Course of Research:

We will initiate studies to determine whether TAG-72 is a proteoglycan or a mucin. These studies will include physiochemical studies as well as enzymatic digestions to determine whether the antigen contains protein and/or carbohydrate components.

Studies will also be initiated to examine the nature of the antigen detected by MAb B72.3 in various tumors. Studies will be performed using both primary and metastatic tumors from breast, colon and ovarian cancer patients to determine

if the antigen detected by B72.3 in these tumors is identical. Since the TAG-72 antigen is secreted, we will also determine the nature of the antigen in serum, ascites and pleural effusions from patients with breast, colon or ovarian cancer.

We have established a competitive radioimmunoassay for the detection of TAG-72 in human tumor extracts and sera based on inhibition of the binding of B72.3 IgG to an extract that contains TAG-72. While this assay has the desired sensitivity and specificity, the long incubation times make it cumbersome for the screening of large numbers of samples. We will therefore attempt to establish a multi-determinant RIA for the detection of TAG-72. Once this assay is developed we will use it to screen a limited number of sera from patients with colon, breast and ovarian carcinomas as well as patients with benign tumors and other diseases of these organs. Sera from patients with carcinomas of other sites as well as other types of malignancies such as leukemias, lymphomas, sarcomas and melanomas and a limited number of normal sera will also be tested for the presence and quantity of TAG-72. The assay for TAG-72 will also be used in collaboration with Dr. Henry Sears (Fox Chase, Philadelphia, PA) to study the serum levels of TAG-72 in patients that are being followed longitudinally. Studying these serum samples will enable us to determine the utility of the assay as a method for detecting recurrence of disease as well as a method for monitoring response to therapy.

In order to understand biodistribution studies of radiolabeled B72.3 in the carcinoma patients undergoing clinical trials, several parameters will be studied including: (1) what effect the presence of circulating antigen will have on the biodistribution of the injected MAb and (2) whether the patients will develop an anti-mouse immune response after injection of the MAb.

Publications:

Colcher, D., Horan Hand, P, Nuti, M., Schlom, J.: A spectrum of monoclonal antibodies reactive with human mammary tumor cells. Proc. Natl. Acad. Sci. (USA) 78: 3199-3203, 1981.

Schlom, J., Colcher, D., Teramoto, Y.A., Horan Hand, P., Nuti, M., Austin, F., Mariani, R., and Wunderlich, D.: Generation and characterization of murine and human monoclonal antibodies reactive with human mammary tumor cells. In Mitchell, M.S. and Oettgen, H.F. (Eds.): Hybridomas in Cancer Diagnosis and Treatment. New York, Raven Press, 1982, Vol. 21, pp. 213-214.

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Herlyn, M., Blaszczyk, M., Sears, H.F., Verrill, H., Lindgren, J., Colcher, D., Steplewski, Z., Schlom, J., and Koprowski, H.: Detection of carcinoembryonic antigen and related antigens in sera of patients with gastrointestinal tumors using monoclonal antibodies in double determinant radioimmunoassays. Hybridoma 2: 329-339, 1983.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09008-04 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*

Localization of Human Tumors in Athymic Mice with Labeled Monoclonal Antibodies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David Colcher	Supv. Microbiologist	LTIB, DCBD, NCI
Jose Esteban	Visiting Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)* F. Mornex and O. Gansow, Radiation Oncology, DCT, NCI; V. Terranova, Laboratory of Developmental Biology and Anomalies, NIDR, NIH; A. Keenan and S. Larson, Department of Nuclear Medicine, CC, NIH; J. Lundy, SUNY, NY, NY

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.9	1.4	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the spece provided.)*

Monoclonal antibodies B6.2 and B72.3 bind to human breast and colon tumor associated antigens. IgG from both these monoclonal antibodies was purified and F(ab')2 and Fab' fragments were prepared from the B6.2 IgG. The B72.3 IgG and B6.2 IgG and fragments were radiolabeled with I-125 and I-131 without loss of their immunoreactivity. The radiolabeled antibodies and fragments were injected into athymic mice bearing antigen positive human breast or colon tumors or an antigen-negative melanoma as a negative control. The B6.2 IgG and fragments localized specifically in the breast tumor xenografts with the IgG giving maximal activity in the tumor. The F(ab')2 fragment gave the best tumor-to-normal tissue ratios (15-20:1 for liver and spleen) due to its rapid clearance from the blood stream. The Fab' fragment cleared more rapidly than the F(ab')2 or the intact IgG with the majority of the radioactivity in the kidneys. When radiolabeled B72.3 IgG was injected into mice bearing colon carcinoma xenografts activity in the tumor rose for the first 2 days and remained constant over the 19 day period of study. Tumor-to-normal tissue ratios rose over this period of time with ratios of approximately 18:1, for liver, spleen and kidney, at 7 days. At 19 days approximately 40% of the radiolabeled B72.3 IgG was found in the tumor. Model systems that resemble the metastatic nature of human colon carcinomas are being developed to better determine the efficacy of radiolabeled monoclonal antibodies as potential agents for radioimmunodetection and radioimmunotherapy.

PHS 6040 (Rev 1/84) GPO 914-916

Project Description

Objectives:

To test the efficacy of monoclonal antibodies as radiopharmaceutical agents in appropriate model systems. To study the parameters for the use of monoclonal antibodies reactive with human mammary and colon carcinoma associated antigens as radioimmunodetection agents. To study the choice of radionuclides, the labeling method and specific activity of the radiolabeled antibody and their effects on the immunoreactivity of the labeled monoclonal antibody in vitro and in vivo. To study the use of radiolabeled IgG and fragments of IgG by various inoculation protocols in model systems to determine their efficacy as radioimmunodetection agents and if successful to initiate studies on the utility of radiolabeled monoclonal antibodies as therapeutic agents.

Methods Employed:

Monoclonal antibodies that react preferentially to human mammary and colon tumor associated antigens were purified from ascitic fluids of mice by ion exchange chromatography and molecular sieving. Pepsin or papain was then used to generate $F(ab')_2$ and Fab' fragments which were then rechromatographed. The IgG and its fragments were radiolabeled with a variety of radionuclides and assayed for immunoreactivity in solid phase radioimmunoassays. The radiolabeled antibodies were injected IV or IP into athymic mice bearing human tumors, and in vivo distribution of the label was determined by analyzing the tissues for radioactivity in the various organs and by scanning of the mice.

Major Findings:

(a) Iodination of IgG and fragments. Monoclonal antibody B6.2 was purified by salt precipitation and ion exchange chromatography. Some of the purified IgG was used to generate $F(ab')_2$ and Fab' fragments by pepsin digestion. The fragments were purified by molecular sieving and retained all their immunoreactivity when compared on a molar basis to the intact IgG. The IgG and its fragments were labeled with ^{125}I using the Iodogen method. Using RIAs, the ^{125}I-labeled IgG, $F(ab')_2$, and Fab' was observed to bind to extracts of a breast tumor metastasis while no binding was detected to extracts from normal human liver, lymphoid cells, or a rhabdomyosarcoma. The labeled antibody was shown to bind to the surface of MCF-7 cells and retained the same specificity as the unlabeled antibody. More than 70% of the antibody remained immunoreactive after labeling. B6.2 IgG and fragments have also been radiolabeled with isotopes that are more useful clinically, namely ^{123}I and ^{131}I.

A great deal of information was obtained from the B6.2 system, but some major differences were observed with monoclonal antibody B72.3. B72.3 IgG was purified and iodinated using the method established for B6.2 IgG. The antibody was efficiently labeled but the immunoreactivity was greatly reduced. Studies using a variety of other labeling techniques were undertaken using chloramine-T, lactoperoxidase and Bolton-Hunter reagent. The IgG was efficiently radiolabeled using all of the methods, but the immunoreactivity of the antibody was greatly reduced in all cases. The Iodogen method was therefore examined closely and after adjusting the 3 major parameters, i.e., ratios of immunoglobulin protein, iodine, and Iodogen, a protocol was obtained [40 ug of IgG, 0.5 mCi of $Na^{125}I$,

20 ug of Iodogen] that yielded a labeled antibody that would bind over 80% of its radioactivity to tumor extracts as measured in sequential solid-phase RIAs.

The iodinated monoclonal antibody bound well in a RIA to the breast tumor metastasis that was used as the immunogen while showing no reactivity to another breast tumor metastasis which was previously shown to lack TAG-72. No binding of B72.3 was observed to apparently normal liver or a normal human lymphoid cell line. The radiolabeled antibody was then tested for reactivity to a number of human tumor lines in order to develop a model for radioimmunodetection studies with the B72.3 antibody. ^{125}I-B72.3 exhibits substantial reactivity with a colon carcinoma tumor (LS-174T) grown in athymic mice, while no binding was observed to extracts of the A375 melanoma xenograft which was shown previously to lack the TAG-72 antigen.

(b) Tumor distribution studies with B6.2. We have shown that we can radiolabel B6.2 IgG and maintain its immunoreactivity. We therefore studied a variety of model systems in athymic mice, including human breast tumor xenografts established from cell lines and from transplantable breast tumors. Approximately 10-20 days after athymic mice were trocared with pieces of a transplanted human mammary tumor (Clouser) the tumors grew to detectable nodules. While the growth rate of the tumors varied as did the final size obtained (0.5 to 2.5 cm in diameter), this model proved more useful than the other models we studied. Athymic mice were also injected subcutaneously with cells from a human antigen-negative melanoma cell line (A375).

Athymic mice bearing the Clouser human mammary tumor were given injections of 0.1 ug of B6.2 IgG labeled with ^{125}I. The ratio of radioactivity per mg in the tumor compared to that of various tissues rose over a 4-day period and then fell at 7 days. The tumor:tissue ratios were 10:1 or greater in the liver, spleen, and kidney at Day 4. Ratios of the cpm in the tumor to that found in the brain and muscle were greater than 50:1 and as high as 110:1. Lower tumor:tissue ratios were obtained to blood and the lungs (with its large blood pool). The absolute amount of ^{125}I-labeled IgG found in the tumor ranged from 2 to 15% of the injected dose, depending on tumor size.

When the Clouser mammary tumor-bearing mice were given injections of ^{125}I-F(ab')$_2$ fragments of B6.2, higher tumor:tissue ratios were obtained. The tumor:tissue ratios in the liver and spleen were 15:1 to 20:1 at 96 hr. The tumor:tissue ratios were somewhat lower with blood and lungs but were still higher than those obtained using IgG. This is probably due to the faster clearance of the F(ab')$_2$ fragments as compared to the IgG. The tumor:kidney ratios were relatively low and were probably a result of the more rapid clearance. The absolute amount of the ^{125}I-F(ab')$_2$ fragments in the tumor ranged from 1 to 12% of the injected dose, depending on tumor size, with a decrease in radioactivity per mg over the 4-day period.

Athymic mice bearing a human melanoma (A375), a tumor that shows no surface reactivity with B6.2 in live cell RIAs, were used as controls for nonspecific binding of the labeled antibody or antibody fragments to tumor tissue. No preferential localization of the monoclonal antibody was observed in the tumor; in fact, the cpm per mg in the tumor were lower than that found in many organs resulting in ratios of <1. Similarly, no localization was observed when either

normal murine IgG or MOPC-21 IgG_1 (the same isotype as B6.2) from a murine myeloma, or their $F(ab')_2$ fragments was inoculated into athymic mice bearing Clouser mammary tumors or melanomas.

Athymic mice bearing Clouser mammary tumors were also given injections of ^{125}I-labeled B6.2 Fab'. The clearance rate of the Fab' fragment was considerably faster than that of the larger $F(ab')_2$ fragment and the intact IgG. Acceptable tumor:tissue ratios were obtained, but the fast clearance rate resulted in a large amount of the labeled Fab' being found in the kidney and bladder, resulting in low tumor:kidney ratios. These studies therefore indicate that $F(ab')_2$ fragments were superior to Fab' or intact IgG in the radioimmunolocalization studies with monoclonal antibody B6.2.

The blood clearance studies following injection of ^{125}I-labeled B6.2 IgG, $F(ab')_2$ and Fab' into nude mice bearing Clouser tumors were performed. As expected, the smaller Fab' fragment cleared more rapidly than the $F(ab')_2$ fragment which in turn cleared more rapidly than the intact IgG. While molecular size probably accounts for this behavior, it is also possible that the smaller fragments are dehalogenated more rapidly, which could also accelerate clearance of ^{125}I activity from the blood pool. The slow components for the blood clearance of B6.2 IgG, $F(ab')_2$ and Fab' are 41 hr, 14 hr, and 4 hr, respectively. Another way of expressing blood clearance data, perhaps more relevant to a potential imaging application is the calculation of the time at which the activity in the blood pool has decreased to 10% of its initial value. For ^{125}I-labeled B6.2 IgG, $F(ab')_2$ and Fab', this occurs at 69 hr, 32 hr, and 4 hr, respectively.

(c) Imaging of Xenografts in Athymic Mice with B6.2. Studies were undertaken to determine whether the localization of the ^{125}I-labeled antibody and fragments in the tumors was sufficient to detect using a gamma camera. Athymic mice bearing the Clouser mammary tumor or the A375 melanoma were given i.v. injections of approximately 30 uCi per 5 ug of ^{125}I-B6.2 IgG. The mice were imaged and then sacrificed at 24 hr intervals. The Clouser tumors were easily detected at 24 hr using radiolabeled B6.2 IgG, with a small amount of activity detectable in the blood pool. The tumor remained strongly positive over the 4-day period, with the background activity decreasing to the point where it was barely detectable at 96 hr. No tumor localization was observed using radiolabeled B6.2 IgG in the mice bearing the control human melanoma transplants of similar size. Mice bearing Clouser mammary tumors or melanomas were also given injections of normal murine IgG radiolabeled with ^{125}I; the scanning data demonstrated no specific localization and were consistent with the tissue distribution data given above.

Mice were also given injections of ^{125}I-B6.2 $F(ab')_2$ fragments. The mice cleared the fragments faster than they cleared the intact IgG, and a significant amount of activity was observed in the 2 kidneys and bladder at 24 hr, but tumors were clearly positive for localization of the ^{125}I-B6.2 $F(ab')_2$ fragments. The activity was cleared from the kidneys and bladder by 48 hr, and the tumor:background ratio increased over the 4-day period of scanning, with little background, and good tumor localization was observed at 96 hr. No localization of activity was observed with the radiolabeled B6.2 $F(ab')_2$ fragments in the

athymic mice bearing the A375 melanoma, nor was any localization observed using normal murine $F(ab')_2$ in mammary tumor-bearing mice. While B6.2 $F(ab')_2$ fragments appeared to be best for radioimmunodetection, a smaller percentage of the injected dose was retained in the tumor as compared to the IgG. For therapy studies the intact IgG may be better because it will deliver a greater dose to the tumor.

(d) Tumor Distribution Studies with B72.3. Radiolocalization studies were also performed with B72.3 using athymic mice bearing human colon carcinomas (LS-174T) in comparison with a human melanoma xenograft (A375) as an antigen-negative control for nonspecific uptake of immunoglobulin. Athymic mice were given s.c. injections of 4×10^6 cells. Tumor growth was rapid with a doubling time of approximately 2 to 3 days for the LS-174T cells. After 7 to 10 days when the tumors were approximately 0.3 to 0.5 cm in diameter, the mice were given i.v. injections of approximately 1.5 uCi of ^{125}I-B72.3 IgG or ^{125}I-MOPC 21 IgG (control antibody of the same isotype). The ratio of cpm radioactivity:mg of tissue in the LS-174T tumor in comparison with that of various tissues was examined over a 7-day period. The tumor:tissue ratio rose over this period with tumor:liver, tumor:spleen, or tumor:kidney ratios of approximately 18:1 at Day 7. Tumor:blood ratios also rose during this time, resulting in ratios of 5:1 at Day 7. There was no specific uptake of ^{125}I-B72.3 IgG in any of the normal organs examined. Approximately 10% of the injected dose per gram reached the tumor at Day 2 post-inoculation of the radiolabeled antibody.

A major difference between the B6.2 system and the B72.3 system is that the amount of the B72.3 radiolabel at the tumor stayed constant over a longer period of time; the activity on a per gram basis then began to drop as the tumor progressed in size. The increased tumor:tissue ratios result primarily from the clearance of labeled IgG from the blood pools. The absolute amount of radioactivity in the tumor rose over the first 2 days and then remained constant through Day 7. Athymic mice bearing melanomas (A375, a tumor line that shows no reactivity with B72.3 in live cell RIAs) were used as controls; no specific uptake of ^{125}I-B72.3 was observed in the tumors of these control animals. Similarly, no localization was observed in athymic mice bearing the colon carcinoma cell line when using ^{125}I-MOPC 21 IgG as a control antibody.

(e) Imaging of Xenografts in Athymic Mice with B72.3. Studies were then undertaken to determine whether localization of the ^{125}I-labeled B72.3 was sufficient to detect by gamma camera scanning. Athymic mice bearing colon carcinomas or melanomas were given injections of approximately 70 uCi (approximately 5 ug) of ^{125}I-B72.3 IgG; the higher dose was used to minimize imaging time. The mice bearing the human colon carcinomas demonstrated significant tumor uptake at early time points with most of the activity in the area of the tumor. The remaining activity in the mice was detected primarily in the area of the heart and lungs. No significant activity was seen in the liver, kidneys, bladder, or stomach. The lack of activity in these organs indicates that there is no signficant breakdown of the radiolabeled antibody, nor is there a large amount of free iodine due to the deiodination of the antibody. At 48 hr, the activity was still seen primarily in the tumor, with the activity in the area of the heart and lungs significantly decreased. A similar pattern was seen at 72 hr with a continuing decrease in the activity in the vital organs. The proportion of the activity found in the tumor continued to increase. Several mice bearing the control tumor, the A375 melanoma, at the same site as the LS-174T tumors were

imaged at similar time points. No significant activity of the ^{125}I-B72.3 was detected in the area of the melanomas. The activity was primarily seen in the area of the heart and lungs.

Even better images were obtained in the athymic mice bearing the colon carcinoma inoculated with ^{125}I-B72.3 at later time points. The background activity cleared from the mice with a $T_{1/2}$ of approximately 7.5 days. The activity in the tumor stayed essentially constant over the 19-day period studied, while the activity in the rest of the body decreased significantly. No accumulation of activity was seen in any organ. The amount of ^{125}I-B72.3 IgG in the colon carcinomas rose from 7% of the total activity in the mouse at Day 1 to approximately 40% at Day 19.

(f) Development of Model Systems for Human Metastatic Disease. The athymic mouse has been shown to be an excellent recipient for human colon tumor xenografts. However, the metastatic rates of human cancers grown in the nude mouse have been generally low. Because of the ability of B72.3 to localize in the LS-174T tumor xenografts, studies were undertaken to establish metastatic models from this cell line to further study the efficacy of B72.3 to detect visceral tumors and metastases and its efficacy as a potential therapeutic agent. Histologically, the LS-174T tumor in vivo grows as a moderately well differentiated adenocarcinoma. Although the tumor appears to grow encapsulated and compress surrounding normal tissue, tumors greater than 1 cm show evidence of microscopic invasion beyond the "pseudocapsule"; blood vessel invasion can also be demonstrated in some tumors. Because there was growth of the primary tumor beyond the capsule, we looked at the effect of early and late excision of the subcutaneous primary tumor on the development of pulmonary metastases. Tumors were implanted in the flank and, after excision of the primary tumor, ipsilateral axillary and inguinal nodes were examined for metastases, as well as lungs and liver. We were concerned primarily with the development of gross metastases that may be used for radioimmunodetection and therapy studies. In the unmanipulated control group of mice followed for approximately 7 weeks (primary tumor size >2 cm in all mice), two manifested micrometastases in the lung.

A total of 18 mice underwent excision of the primary tumor at approximately 5 mm in size (early excision group). Two of eighteen in this group had a local recurrence. The 16 mice who had no local recurrence had no evidence grossly or microscopically of pulmonary, axillary or inguinal metastases upon sacrifice. Of 26 mice who had late excisions (tumor $\geq$ 1 cm), 23 had local recurrence. Many of these tumors were adherent to the skin and to fascia of the underlying muscle. The 23 who had local recurrences developed them from 7 to 14 days after excision of the primary. Three of the 12 mice sacrificed at 2-3 weeks had gross pulmonary metastases. Five of the 11 mice sacrificed at 4 to 5 weeks after excision of the primary had gross pulmonary metastases which were confirmed microscopically. Once local recurrence developed it grew quite rapidly and precluded longer followup. It appears, therefore, that the growth of the primary tumor is too rapid to permit the generation of gross metastases. The animals develop large necrotic primary masses before any metastases are established. Only by removing the primary tumor and allowing it to recur will metastases be found. The recurrence of the primary tumor, however, precludes the use of this model for localization or therapy studies of metastases.

The site of primary tumor was therefore changed in an attempt to gain better local control, with the hope that we still might be able to increase the metastatic rate if we could follow the mice for an extended period of time. The LS-174T tumor was placed as a single cell suspension in the hind foot pad. The tumors grew more slowly and did not reach 1 cm in size for at least 4 weeks. Amputation of the leg, to preclude the recurrence of the primary tumor, was performed at that time. Six to eight weeks post-amputation, mice were sacrificed. At eight weeks post-amputation 42% of mice had pulmonary metastases. While this model provided metastases, their relatively low frequency and the difficulty of the model precludes its use as a therapy model.

Due to absence of intraabdominal metastases from subcutaneous primary sites, we attempted direct implantation of tumor cells in the spleen to establish intraabdominal visceral growth. It was postulated that tumor cells might metastasize to the liver via the portal venous circulation, thus resembling the spread of a human colon carcinoma. A dose/response curve demonstrated that a dose of $1x10^5$ cells was necessary to establish tumor growth in the spleen with a success rate of approximately 80%. Of 12 mice bearing splenic tumors, 3 were found to have lung metastases (2 gross and 1 microscopic). Two of 24 mice had gross liver metastases. This model while giving a relatively low frequency of metastases proved useful in providing tumors on visceral organs which is more representative of the human disease.

Significance to Biomedical Research and the Program of the Institute:

Radiolabeled monoclonal antibodies reactive with the surface of human carcinoma cells may prove useful in the diagnosis and management of human cancer. The use of radiolabeled monoclonal antibodies in lymphoscintigraphy of the internal mammary chain as well as the axillary nodes may increase the reliability of staging of nodal involvement as a prognostic indicator. The detection of occult metastatic lesions at distal sites via gamma scanning for many types of cancer could serve as an adjunct in determining which patients should receive adjuvant therapy, while subsequent scanning could reveal which tumors are responding to therapy. Monoclonal antibodies can also be used as therapeutic agents. The use of monoclonal antibodies coupled with isotopes decaying via high-energy transfer with short-range radiation could prove useful as radiotherapeutic agents.

Proposed Course of Research:

We have shown that monoclonal antibodies, radiolabeled with ^{125}I or ^{131}I, can localize in human tumor xenografts in athymic mice. The goals of this project are: (1) to define the parameters necessary to enhance the ability of radiolabeled MAbs to detect and image tumor xenografts, and (2) to define those parameters for the successful use of monoclonal antibodies as therapeutic agents.

While some antibodies were able to be fragmented using established protocols, others were degraded completely. B72.3 IgG, when treated with pepsin, is degraded to yield a non-immunoreactive fragment. We will, therefore, examine the utility of a variety of enzymes and techniques to determine if a suitable fragment can be obtained.

We have shown that we can radioiodinate MAb IgG and fragments with ^{125}I and ^{131}I without loss of their immunoreactivity. ^{125}I is not applicable in human studies because of its low energy. ^{131}I can be used for clinical studies, but its high energy gamma emissions are not easily detected by gamma cameras and its beta emissions result in a significant radiation dose to the patients. There are several radionuclides that can be used for imaging studies that have more suitable emission properties. These include ^{123}I, ^{111}In and ^{99m}Tc. We plan to use B72.3 IgG and its fragments, when available, to study the utility of these coupled radionuclides as imaging agents in model systems.

We have recently developed new monoclonal antibodies to a number of tumor-associated antigens. Studies will be undertaken using competitive binding assays to determine which of these antibodies bind to different determinants in order to develop a subset of antibodies for use as radioimmunodetection agents. These antibodies will then be used in pairs with each other and with B72.3 to determine if "cocktails" of antibodies are more efficient in binding to human tumor xenografts in athymic mice.

We have shown that subcutaneous human colon carcinoma xenografts in athymic mice can be used for localization and therapy studies with radiolabeled monoclonal antibodies. The subcutaneous model while very useful does not mimic the disease in humans. The tumors grow locally and rarely metastasize. We are establishing models for intra-peritoneal (IP) disease using the human colon carcinoma cell line LS-174T. We are planning on developing another model in collaboration with Dr. V. Teranova in an attempt to select metastatic tumor cells. This model may be most representative of the human disease in that tumors form in a variety of tumor sites including lymph nodes.

The use of monoclonal antibodies as therapeutic agents is a major goal of the in vivo studies and is one of our long term goals. Before undertaking therapy trials it is necessary to demonstrate that a monoclonal antibody is reaching its target cell specifically. When this has been accomplished we will couple to MAb B72.3 radionuclides that can deliver toxic doses and examine the utility of these radiopharmaceuticals as in vivo therapeutic agents.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09018-01 LTIB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Clinical Trials with Radiolabeled Antibodies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jeffrey Schlom	Chief	LTIB, DCBD, NCI
David Colcher	Supv. Microbiologist	LTIB, DCBD, NCI
Jose Esteban	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

S Larson, Chief, Nuclear Medicine, NIH
P. Sugarbaker, Chief, Colorectal Surgery, Surgery Branch, NCI
G. Bryant, Laboratory of Pathology, NCI

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.8	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Accurate detection and anatomic localization of both primary and metastatic lesions remains one of the major problems in the management of most human carcinomas. We have recently initiated clinical trials at the NIH Clinical Center to detect and localize colorectal carcinoma lesions using radiolabeled MAb B72.3. Parameters that will be systematically investigated concerning both the efficiency of MAb localization and the efficiency of gamma scanning of carcinoma lesions include: (a) effect of MAb dose and specific activity of radionuclide coupled MAb; (b) comparison of the use of intact IgG, $F(ab')_2$, and Fab'; (c) choice of radionuclide;(d) route of inoculation; (e) size, location, and other inherent properties of the tumor mass such as antigen content; (f) the presence of circulating antigen; (g) the presence and/or absence of human anti-murine Ig antibodies; (h) metabolism of MAb and fragments; (i) combinations of MAbs. It is hoped that these studies will also aid in establishing a rational basis for the subsequent therapeutic use of a particular MAb, either coupled to toxins, via effector cell-mediated or complement-mediated mechanisms, or using MAbs radiolabeled with one of a variety of isotopes. This latter goal can be accomplished by direct analyses of biopsy material (both tumor and normal tissues) from patients receiving radiolabeled MAb to define the "radiolocalization index" or potential "therapeutic index" (i.e., the ratio of the amount of MAb bound [via cpm] per gram of tumor tissue to that bound per gram of normal tissues).

GPO 914-918

Project Description

Objectives:

We plan to systematically investigate the effect of the following parameters on the efficiency of MAb localization and the efficiency of gamma scanning of carcinoma lesions including: (a) effect of MAb dose and specific activity of radionuclide coupled MAb; (b) comparison of the use of intact IgG, $F(ab')_2$, and Fab'; (c) choice of radionuclide;(d) route of inoculation; (e) size, location, and other inherent properties of the tumor mass such as antigen content; (f) the presence of circulating antigen; (g) the presence and/or absence of human anti-murine Ig antibodies; (h) metabolism of MAb and fragments; (i) combinations of MAbs. It is hoped that these studies will also aid in establishing a rational basis for the subsequent therapeutic use of a particular MAb, either coupled to toxins, mediated via effector cell-mediated or complement-mediated mechanisms, or using MAbs radiolabeled with one of a variety of isotopes. This latter goal can be accomplished by direct analyses of biopsy material (both tumor and normal tissues) from patients receiving radiolabeled MAb to define the "radiolocalization index" or potential "therapeutic index" (i.e., the ratio of the amount of MAb bound [via cpm] per gram of tumor tissue to that bound per gram of normal tissues).

Methods Employed:

Monoclonal antibodies are purified by ion exchange chromatography and molecular sieving. Several enzymatic treatments are used to generate $F(ab')_2$ and Fab' fragments of IgG. The intact IgG and fragments are radiolabeled with various radionuclides and assayed for immunoreactivity. Several radioimmunoassays are employed to assay for free and tissue bound radiolabeled antibody, immune complexes, human anti-murine Ig antibodies, and serum antigen levels.

Major Findings:

We have recently initiated a clinical trial at the NIH Clinical Center using radiolabeled MAb B72.3 entitled "The Use of Radiolabeled Monoclonal Antibody to Detect Colorectal Carcinoma Metastases." This trial is being conducted in close collaboration with Dr. Paul Sugarbaker (Surgery Branch, NCI), Dr. Steven Larson (Nuclear Medicine, NIH), and Dr. G. Bryant (Pathology Branch, NCI).

One obstacle that had to be overcome prior to consideration of the clinical trial was: (a) preparation of clinical grade and vialed drug (i.e., purified MAb B72.3 IgG, and ^{131}I radiolabeled MAb B72.3 IgG). Protocols were established in which all glassware and reagents (buffers, columns, chromatography material, etc.) had to be tested for proof of being sterile and pyrogen free. Each lot of vialed MAb must be evaluated for, and shown to be: (a) sterile, (b) free of pyrogen, (c) free of twelve adventitious murine viruses by immunoassays, (d) negative for murine retrovirus by the S+L- test, (e) tested for general safety and toxicity by inoculation into rodents (f) free of detectable murine DNA, (g) free of *Mycoplasma* and (h) efficiency of binding.

The protocol for the detection of colon carcinoma metastases using ^{131}I-MAb B72.3 can be summarized as follows: 1. Patients are usually those who have failed conventional therapy for metastatic colon cancer and have entered the NCI Surgery Branch Protocol for the surgical resection of tumor metastases followed by administration of chemotherapy. Thus, all patients inoculated with ^{131}I-MAb B72.3 will, approximately eight days later, have metastatic tumor masses resected in addition to having multiple biopsies of normal tissues (the "normal" tissue is removed for staging purposes, i.e., to be examined for micrometastases). This permits the direct examination of biopsy specimens and thus an accurate determination as to where the MAb localizes, and in what amounts. 2. Sections of formalin fixed primary tumors or metastases (when available) that were previously removed are examined using the ABC immunoperoxidase method and MAb B72.3 to determine if any correlations exist between TAG-72 antigen expression in primary masses and metastatic lesions subsequently removed. 3. Approximately eight days prior to patient surgery, MAb B72.3 is radiolabeled with ^{131}I, purified by column chromatography, and tested for TCA precipitability, lack of pyrogenicity (by an LAL assay), and specificity and efficiency of binding to a standard TAG-72 positive tumor extract and lack of binding to an extract of normal human liver. 4. Approximately eight days prior to surgery, patients are administered ^{131}I-MAb B72.3 intravenously (I.V.) and scanned with a gamma camera at 2 hours and daily until surgery. Patients were also monitored for total body clearance rates via gamma scanning. 5. Serum samples are obtained prior to inoculation with MAb and at the following times post inoculation: 5, 30, 60, 120 and 240 minutes, and daily for seven days. Serum samples will be analyzed for: (a) TAG-72 antigen levels by RIA, (b) human anti-murine antibodies via RIA (samples taken up to 3 months), (c) TAG-72 antigen/^{131}I-MAb complexes via high performance liquid chromatography (HPLC), (d) ^{131}I-MAb/human anti-murine antibody complexes via HPLC, and (e) level of cpm per ml of blood (for isotope clearance). Urine sample are obtained for 6 days post inoculation for analysis of radioactivity per ml and total cpm, i.e., ^{131}I clearance. 6. At surgery, tumor and "normal" tissues are removed and sent to Pathology. Representative samples of individual tissues are immediately weighed and analyzed for cpm of ^{131}I per gram of tissue, as well as sectioned for analysis by autoradiography. Five micron sections will be analyzed for (a) percent tumor cells of total cells present, and (b) percent of tumor cells that are positive for the TAG-72 antigen using the ABC immunoperoxidase method and MAb B72.3

To date, 17 patients have received ^{131}I-labeled MAb B72.3 and preliminary findings are outlined in Table 1. The amount of B72.3 IgG administered, the level of mCi coupled to the IgG, and specific activities (mCi^{131}I per mg MAb IgG) are given. To date, two dose levels of MAb IgG have been employed (approximately 1-2mg or 8-10mg). The specific activities used can be placed into 3 groups: (i) approximately 0.4 mCi of ^{131}I/mg of IgG, (ii) 2-3 mCi/mg, and (iii) 7-10 mCi/mg MAb. It should be emphasized that these results are being presented as preliminary findings at this time, and thus each group in terms of dose or specific activity is still small. Clinical toxicity of any kind has not been observed in any of the patients who have received radiolabeled MAb B72.3. Gamma scans accurately identified tumor masses prior to surgery in 9 of the 17 patients. Two points are

emphasized here: (a) a "positive" represents an identification of a tumor mass prior to surgery and (b) anatomic localization and size of tumor mass was confirmed at the time of surgery. Thus localization of the tumor and tumor measurements were those obtained at surgery or immediately post operatively by gross pathology. Perhaps the most important findings of these preliminary studies are the tumor to normal tissue ratios obtained from the surgical specimens. As seen in Table 1, MAb B72.3 IgG selectively localized (in terms of cpm per gram of histologically confirmed carcinoma versus cpm bound per gram of histologically confirmed normal tissue) to the vast majority of tumor masses in all patients. In most patients (12 of 14) tumor to normal tissue MAb localization ratios ranged from 2:1 to, in most cases from 5:1 to as much as 38:1.

Significance to Biomedical Research and the Program of the Institute:

Accurate detection and anatomic localization of both primary and metastatic carcinoma lesions remains one of the major problems in the management of colon, breast, and ovarian carcinomas. Earlier detection of both primary and metastatic lesions, with subsequent earlier intervention by conventional therapeutic modalities may well lead to both better quality of life and longer survival among carcinoma patients. The use of radiolabeled MAbs directed against tumor associated antigens (TAAs) may thus be of use toward the following goals: (a) earlier detection of primary lesions, (b) more accurate staging, in particular, determining the involvement of regional nodes, (c) detection of the presence of distal metastases at the time of surgery of the primary lesion, (d) monitoring the efficiency of conventional therapy by sequential analysis of tumor size, (e) detection of metastases in patients during the 5-10 year period following removal of primary mass and (f) establishing a rational basis for the subsequent use of a particular MAb.

Proposed Course of Research:

The present protocol as described in the "Major Findings" section using ^{131}I-labeled MAb B72.3 IgG to detect metastatic colon carcinoma will be continued and modified to systematically determine answers to the following questions, and systematically define the following parameters: (1) effect of MAb dose, amount of coupled radionuclide and specific activity of radiolabeled MAb on both tumor visualization and "radiolocalization index" of the MAb; (2) comparison of MAb B72.3 IgG, F(ab')$_2$, and Fab' on tumor localization; (3) choice of radioisotope; (4) route of inoculation; (5) effect of tumor size, location and histologic characteristics on MAb localization and gamma scanning; (6) presence of TAG-72 antigen in the serum; (7) presence of human anti-murine Ig antibodies; (8) total body clearance, blood clearance, and renal clearance of radiolabeled MAb and fragments; and (9) antigen content of tumor.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 05216-14 LTIB
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October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
cAMP Binding Proteins in Mammary Cancer Growth Control

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Y.S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBD, NCI
T. Clair	Chemist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)* C. L. Kapoor, Retina Foundation Fellow, LVR, EI, NIH
Dr. W. R. Miller, U. of Edinburgh; F. L. Huang, Expert, LCCTP, NCI, NIH
Dr. S. O. Doskeland, U. of Bergen, Norway
Dr. J. D. Corbin, Howard Hughes Medical Inst. Lab., Vanderbilt U., Nashville, TN

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Laboratory of Tumor Immunology and Biology

SECTION
Cellular Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have demonstrated that cyclic adenosine 3', 5'-monophosphate (cAMP) and its binding proteins are involved in the regulation of the growth of mammary tumors in experimental animals. Whilst human breast cancers which possess estrogen receptor (ER) and progesterone receptor (PgR) activities are likely to be hormone responsive, many do not respond to endocrine therapy. In hormone-dependent rat mammary tumors the ratio of steroid receptors to cAMP binding proteins was found to better discriminate hormone-dependent from independent tumors than steroid receptor alone.

In this study we investigated the relationship between cAMP binding proteins, ER and PgR in human breast cancers and clinical parameters including prognosis. Several molecular species as well as proteolytic fragments of cAMP binding proteins have been found in normal and neoplastic tissues. The molecular species of cAMP binding proteins were determined by the photo-incorporation of 8-azido-[^{32}P] cAMP and the immunopercipitation using affinity purified antibodies to cAMP binding proteins. Utilizing immunocytochemical method intracellular distribution and nuclear compartmentalization of cAMP binding proteins were also determined. Finally, in a cell-free system, the binding of cAMP binding proteins directly to DNA of normal vs cancer cells was studied. The goal of this proposal is to elucidate the fundamental growth regulatory mechanism of cAMP which can be applied to the breast cancer therapy.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To assess the relationship between the ratio of steroid receptors to cAMP binding proteins and hormone-dependency of breast cancer in humans. To determine the molecular species and intracellular compartmentalization of cAMP binding proteins before and after cAMP treatment. To assess DNA binding of cAMP binding proteins in a cell-free system.

Methods Employed:

1. Tumors: Primary and metastatic tumors from patients with breast cancer and primary, 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary carcinoma and transplantable MTW9, DMBA #1, MT13762, mammary carcinomas in rats were used.

2. MCF-7 cells: The MCF-7 human breast cancer cells (Mason Research Institute) were grown in McCoy's 5A medium supplemented with bovine insulin, penicillin, streptomycin and fetal calf serum (10%) and ± additives.

3. cAMP assay: cAMP was measured by the competitive protein-binding method of Gilman using cAMP assay kit of Amersham.

4. Protein kinase assay: The activity was determined by measurement of the incorporation of ^{33}P from γ-labeled ATP into histone ± 10^{-6}M cAMP.

5. Estrogen-binding assay: Estrogen-binding was measured by the modification of the charcoal adsorption assay described originally by Korenman.

6. cAMP-binding assay: cAMP-binding activity was measured by competion assay of Gilman at pH 6.5 at 23° for 3 hr (^{3}H cAMP-binding to proteins includes not only free sites but also endogenously bound sites by the exchange). The binding reaction was stopped by the addition of ice-cold 3.6 M ammonium-sulfate (pH 7.2) to precipitate protein-bound [^{3}H]cAMP. The precipitates were collected on membrane filter and the radioactivity measured by liquid scintillation.

7. Photo-affinity labeling of cAMP binding protein: The photo-affinity incorporation of 8-N_3-[^{32}P]cAMP was performed by the method of Pomerantz et al. (Biochemistry, 14: 3858, 1975), the binding proteins labeled with the 8-N-[^{32}P] cAMP were analyzed by SDS-PAGE.

8. Antibodies: cAMP-binding proteins (R^{I} and R^{II}) were purified from the bovine skeletal muscle and bovine heart. Antibodies were raised in rabbits. The antibodies were affinity purified using a glutaraldehyde cross-linked immunoabsorbent technique. The monospecificity of antibody was confirmed. The cross-reactivity of R of MCF-7 cells with bovine anti-R^{I} and -R^{II} was demonstrated by radioimmunoassay (Kapoor and Cho-Chung, Cancer Res. 43: 295, 1983).

9. Immunocytochemistry: Unfixed cryostat sections (≈4μ) of tumors were used for the indirect immunofluorescence cytochemistry of cAMP binding

proteins (R^I and R^{II}). The sections are examined with a Zeiss epifluorescence microscope.

10. DNA-binding of cAMP-binding protein, R^{II}: DNA-binding of R^{II} will be performed using a cell-free system of DMBA-induced mammary tumor (Science 205: 1390, 1979), followed by DNA-isolation and agarose gel electrophoresis.

Major Findings:

Prognostic value of cAMP bindng proteins in human breast cancer: Cyclic AMP binding activity was measured in the cytosols from 75 human breast cancers. All tumors contained measurable binding proteins, levels varying from 0.81 to 15.05 pmol/mg cytosol protein (mean=5.34). No relationship was found between level of cAMP binding activity and menopausal status of the patient, clinical stage of the disease, presence or absence of nodal involvement or histologicl grade of the tumor.

A group of 11 patients who at the time of primary treatment had no evidence of metastatic disease but have developed recurrence within 36 months have been compared with a similar group (10 patients) who have had a minimum of 20 months follow-up and were disease-free. Levels of cAMP binding proteins were significantly higher in tumors from women having early recurrence than in those who were disease-free ($p<0.01$ by Wilcoxon and Rank Test). The range in disease-free patients was 1.57-7.21 pmol/mg cytosol protein; whereas that in 10 of 11 tumors which subsequently recurred early was 7.75-13.02. It is concluded that although levels of cAMP binding proteins are not associated with the clinical parameters described, it may be of independent prognostic significance.

Relationship of hormone sensitivity to estrogen receptor and cAMP binding capacity in human breast cancer: Estrogen receptor (ER) and cAMP binding capacity [cAMP receptor (CR)] were measured in cytosols from human breast tumors. Patients with advanced, evaluable breast cancer were biopsied before start of endocrine treatment, and ER and CR measurements performed. All patients included in this study were ER positive. Sixteen of 30 patients (53%) had an objective response to endocrine treatment. When ER and CR were expressed as a ratio and this ratio was related to treatment response, it was found that 11 objective responders had ratio values above 2.5×10^{-3}. Nine of 14 non-responders had ER/CR ratios below this value. In this study, a threshold limit of 2.5×10^{-3} (ER/CR) would have predicted a correct response to endocrine treatment in 25 of 30 patients (83%). The results show that measurement of cAMP bindng proteins might strengthen the predictive value of steroid receptor measurement for hormone-dependency of human breast cancer.

Nuclear translocation of cAMP + receptor complex triggers tumor regression: In regressing mammary tumors (produced with DBcAMP, Ovex or tamoxifen), a translocation of cAMP + receptor complex occured within the nucleus followed by phosphorylation of a 76,000-dalton nuclear protein. Tumors that failed to show these nuclear events did not regress. It is proposed that the nuclear translocation of cAMP receptor complex is an indispensable triggering event for mammary tumor regression.

By immunocytochemical technique, the cAMP receptor (R^{II}), type II cAMP-dependent protein kinase regulatory subunit was localized in the nucleoli and mitotic spindles of human breast cancer cells, MCF-7 and MDA-MB-231. During regression of MCF-7 tumor in nude mice following hormone-withdrawal, the intensity of immunofluorescence of R^{II} dramatically increased in the nucleoli. The increased immunofluorescence in the regressing tumor was due to a translocation of the 50,000- and 52,000-dalton R^{II} within the nucleoli: during growth, the 50,000- and 52,000-dalton R^{II} was detected only in the cytoplasm, and the nucleoli contained the proteolytic fragment of R^{II}, 44,000- and 34,000-daltons. Thus, nuclear translocation of the intact cAMP receptor, R^{II} as a critical actor in tumor regression is documented.

New gene expression during tumor regression: The in vitro translated proteins from poly(A) RNAs differed when hormone-dependent mammary carcinomas were compared during their growth and regression. Within 6 hours post ovarietomy of the host rats, the concentration of one protein band (M.W. 20,500) increased and those of two protein bands (M.W. 35,000 and 22,000) decreased in the regressing, as compared to the growing, DMBA tumors. Strikingly, the treatment with DBcAMP of tumor-bearing hosts, which produced tumor regression, also resulted in the same changes in the translated protein pattern as ovariectomy. The changes in the translation products observed in the regressing tumors were reversed when resumption of tumor growth was induced by replenishment of estrogen or cessation of DBcAMP treatment. The results suggested that hormone-regulated growth of mammary tumors is related to specific genetic transcripts and that the antagonistic inter-relation between cAMP and estrogen may be responsible for the differential gene expression observed in the growth/regression of mammary tumors.

The carcinogenic dose of 7,12-dimethylbenz(α)anthracene (DBMA) failed to induce mammary carcinoma in the rats that have received DBcAMP. The anti-carcinogenic effect of DBcAMP correlates with its modulatory effect on gene expression. The 22K M.W. protein was found as a prominent in vitro translation product of the carcinogen susceptible young virgin (50-day-old) gland and this protein markedly decreased in the carcinogen-unsusceptible older virgin (110-day-old) gland. DMBA administration in vivo did not alter the production of the 22K protein in the mammary gland, but when DMBA was fed to the young virgins that had received DBcAMP, the production of the 22K protein was sharply reduced. A possible link between chemical mammary carcinogenesis and the 22K M.W. protein was found.

These results suggest that differential gene expression is involved in growth and regression of mammary tumors and mammary carcinogenesis.

DNA-binding of cAMP binding protein R^{II}: Immunocytochemical localization of R^{II} cAMP binding protein in the nucleoli of MCF-7 cells suggested the role of R^{II} cAMP binding protein in the nuclear event, especially in cell division. DNA-binding of R^{II} was demonstrated using a cell-free system of DMBA-induced mammary tumor. The 8-azido[^{32}P]cAMP was incubated in dark with cAMP-dependent protein kinase type II of bovine heart at 23° for 1 hr then further incubated with isolated nuclei from DMBA tumor at 0° for 1 hr, and photolyzed the complex,

DNA was then isolated using the phenol extraction, and the radioactivity bound DNA was identified by agarose-electrophoresis. It was found that the radioactivity band was not detected on the control DNA obtained from the nuclei incubated with the 8-azido[^{32}P]cAMP only in the absence of the protein kinase. The results indicate covalent binding of R^{II} cAMP binding protein to DNA of mammary carcinoma.

Significance to Biomedical Research and the Program of the Institute:

This study contributes to the understanding of the fundamental growth regulatory mechanism of cAMP action. The measurement of cAMP-binding protein may be an important predictive probe for hormonal sensitivity in human breast cancer. The regulatory role of cAMP-binding protein R^{II} in cell division may be of clinical significance. These results together with our previous studies suggest a therapeutic potential for cAMP analogs in human breast cancer. The use of cAMP analogs may substitute for, or synergize the effects of antiestrogens or some of the cytotoxic agents presently in use.

Proposed Course of Research:

To extend the investigation on the regulatory role of cAMP in breast cancer growth control, the following proposal is made: 1) Assess the relationship between the molecular species of cAMP-binding proteins in human breast cancer and clinical stage of the disease or prognosis. 2. Localize the intracellular distribution of cAMP-binding proteins (R^{I} vs R^{II}) in human breast cancer by immunocytochemical method. 3. Localize the binding sites of cAMP-binding protein (R^{II}) in DNA utilizing molecular biology techniques.

Publications:

Huang, F.L., Hasuma, T. and Cho-Chung, Y.S.: Relationship between the anti-carcinogenic effect of $N^{6},O^{2'}$-dibutyryl cyclic adenosine 3',5'-monophosphate and modulation of gene expression and inhibition of binding of 7,12-dimethylbenz (α)anthracene to DNA of mammary cells. Cancer Res. 44: 1595-1599, 1984.

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Cho-Chung, Y.S.: The role of cAMP in the control of mammary tumor growth. In Hollander, V.P. (Ed.): Hormonally Sensitive Tumors. New York, Academic Press, Inc. (In press)

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Cho-Chung, Y.S., Clair, T., DeBortoli, M.E. and Tagliaferri, P.: Suppression of celular and viral ras oncogene expression by cyclic AMP. In Neiburgs, H.E. (Ed.): Cancer Detection and Prevention. New York, A.R. Liss, Inc. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

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PROJECT NUMBER
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October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Regulatory Mechanism of Oncogene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Y.S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBD, NCI
T. Clair	Chemist	LTIB, DCBD, NCI
P. Tagliaferri	Visiting Fellow	LTIB, DCBD, NCI
R. Bassin	Chief, Biochemistry Oncogenes Section	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)* C. L. Kapoor, Retina Foundation Fellow, LVR, EI, NIH
Dr. M. R. Smith, Roche Inst. of Molecular Biology, Nutley, NJ
Dr. J. D. Corbin, Howard Hughes Medical Inst. Lab. Vanderbilt U., Nashville, TN
Dr. W. Weber, Inst. fur Physiologische Chemie der Universitat, Hamberg, Germany

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SECTION
Cellular Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In spite of the relatively large body of information concerning the molecular structure of retrovirus oncogenes and their specific protein products, little is known about the mechanisms by which they transform cells. Moreover, proto-oncogenes, the cellular counterpart of retroviral oncogenes, have been found in normal cells but their expression is low and the mechanism for this low expression is not known. It is conceivable that substances that increase tumor development by apparently increasing cellular proliferation do so by altering the quantitative or temporal expression of cellular oncogenes. Understanding the cellular mechanisms governing the expression of both viral and cellular oncogenes would therefore provide an insight into the mechanism of neoplastic cell growth and tumor development. Occasionally, tumor cells differentiate spontaenously and then regress completely. It has been suggested that cAMP may be linked with the morphological differentiation of neoplastic cells since treatment of some tumor cells with dibutyryl cAMP, prostaglandin E_1 and inhibitors of cAMP-phosphodiesterase induces irreversible morphological differentiation. That this differentiation may be a reversion of malignancy is supported by the observation that no tumor is produced when these treated cells are inoculated into animals. To investigate factors that affect phenotypic reversion of transformed cells, we have chosen a cell line 433 of NIH 3T3 cells containing the transforming ras gene of Ha-MuSV flanked by LTR of MMTV; the expression of ras gene in 433 cells is therefore controlled by mouse mammary tumor virus promoter (MMTV-LTR) which is under control of glucocorticoid. Thus, the phenotypically normal 433 cells become transformed and produce the ras gene product, p21, only upon addition of glucocorticoid. We also used clone 13-3B-4 of NIH 3T3 cells, the Ha-MuSV DNA transfectant, whose expression of v-ras^H gene is not under control of glucocorticoid. The goal of this study is to investigate the effect of intracellular regulatory factors, such as, cyclic nucleotides, hormones, and growth factors on the MMTV-LTR, and MoLV-LTR regulated expression of ras gene.

Project Description

Objectives:

To investigate the effect of cAMP on the p21 expression and morphology of Ha-MuSV transformed NIH-3T3 cells and to determine the behavior of cAMP receptor proteins (R^I and R^{II}), the regulatory subunits of cAMP-dependent protein kinase (type I and type II).

Method Employed:

1. Cells: clone 433.3; an established cell line of NIH-3T3 cells carrying MMTV-LTR-v-ras^H. Grown on polylysine-coated plastic substrate in media containing only transferrin, insulin and salts. Under these conditions, the cells respond dramatically to the addition of dexamethasone. Flat, contact inhibited monolayers are observed in the absence of hormone; in the presence of increasing concentration of dexamethasone, the cells become round and refractile and float away from the substratum. Clone 13-3B-4; NIH 3T3 clone which had been transfected with Ha-MuSV DNA.

2. Cell culture: 433 and 13-3B-4 cells were carried in Dulbecco's modified Eagle minimum essential medium (DMEM) containing penicillin (100 U/ml) and streptomycin (100 µg/ml) and supplemented with 10% fetal calf serum. For experiments with cAMP, cells were grown in serum-free defined medium [DMEM-HAM's F12 (Nutrient Mixture F12-HAM)(75:25)] supplemented with bovine insulin (5 µg/ml), histidine-HCL (42 µg/ml), glutamine (292 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and HEPES (20 mM, pH 7.3) in the presence or absence of additives.

3. p21 Immunoprecipitation: [^{35}S] methionine-labeled cell lysates were incubated for overnight at 4°C with rat monoclonal antibody against Harvey sarcoma virus p21. The reaction mixture was then incubated for an additional 2.0 hr at 4°C with a 10% suspension of Formalin-fixed Staphylococcus aureus (precoated with rabbit anti-rat immunogloublin G). Immunoprecipitates were collected by centrifugation, washed and analyzed by SDS-PAGE.

4. Photo-affinity labeling of cAMP binding proteins: The photo-affinity incorporation of 8-N_3-[^{32}P]cAMP was performed by the method of Pomerantz et al. (Biochemistry 14: 3858, 1975), the binding proteins labeled with the 8-N_3-[^{32}P]cAMP were analyzed by SDS-PAGE.

5. Immunocytochemistry: An indirect immunocytochemistry of cAMP receptor proteins was carried out as described in (Kapoor and Cho-Chung, Cancer Res. 43: 295, 1983). Affinity purified cAMP receptor protein antibodies were utilized to reduce the background fluorescence.

Major Findings:

cAMP and glucocorticoid antagonism on the p21 ras protein synthesis and phenotypic transformation of 433 cells: An established cell line in which the gene product responsible for the transformed phenotype is under control of a hormone responsive promoter made possible the studies on reverse transformation. 433 cell line of NIH-3T3 cells is a stable MMTV-LTR-v-ras^H gene carrier in which the transforming ras gene is not expressed until glucocorticoid is added. Flat, contact inhibited monolayers are observed in the absence of hormone; in the presence of dexamethasone (10^{-9}-10^{-6}M) the cells become round and refractile and float away from the substratum.

This effect of dexamethasone was blocked when DBcAMP (10^{-4}M - 10^{-3}M), 8-thiomthyl-cAMP (10^{-5}M), 8-Br-cAMP (10^{-5}M) or cholera toxin (10 ng/ml) were added 2 days prior to the addition of dexamethasone (10^{-8} - 10^{-6}M): the cells exhibited flat, contact, inhibited monolayers just as the cells in the absence of dexamethasone. The effect of dexamethasone in conversion of normal phenotype to transformed phenotype occurs between 10^{-9}M and 10^{-8}M concentrations, in close agreement with the binding constant for the glucocorticoid receptor, confirming that the cell phenotype "switch" is in fact mediated by the normal cytoplasmic receptor. This is also true with the effect of cAMP analogs in reversion of the transformed to normal phenotype since the effective concentration of cAMP analogs was in close agreement with the binding constant for the cAMP receptor. These results suggest that steroid and cAMP receptors counteract each other directly or indirectly on a hormone responsive promoter that regulates expression of an oncogene.

Phenotypic alteration of 433.3 cells in response to glucocorticoid hormones has been correlated with the increased synthesis of a 21,000 dalton transforming protein (p21), the product of the v-ras^H gene. Levels of p21 in 433.3 cells were determined by radiolabeling cellular proteins with ^{35}S-methionine, followed by immunoprecipitation with monoclonal antibody #259 directed against Harvey sarcoma virus-encoded p21. A substantial amount of p21 was specifically precipitated from extracts of 433.3 cells treated with dexamethasone. The antibody detected no significant amounts of p21 in cells grown in the absence of dexamethasone. Cells treated with dibutyryl cyclic AMP starting 2 days prior to the addition of dexamethasone or with dibutyryl cyclic AMP alone also did not contain detectable levels of p21. These data demonstrated that the p21 encoded by the viral ras portion of the MMTV-LTR:v-ras^H chimeric DNA was induced by dexamethasone, and that this induction was blocked by dibutyryl cyclic AMP.

Suppression of viral ras oncogene expression in clone 13-3B-4 cells (NIH-3T3) by cyclic AMP: Clone 13-3B-4 of NIH-3T3 cells, which had been transfected with Harvey murine sarcoma virus DNA, was used. When 13-3B-4 cells were grown in the serum free medium containing transferrin and insulin, the cells exhibited a marked phenotypic transformation within 2-3 days of culture: they became round, refractile, and piled up, and eventually floated away from the substratum. However, phenotypic transformation of 13-3B-4 cells was completely blocked when the cells were grown in serum free medium containing dibutyryl cyclic AMP

(DBcAMP) (5 x 10^{-4}M): the cells formed flat, contact inhibited monolayers. Other cAMP analogues, as well as cholera toxin, all inhibited phenotypic transformation in this system. This DBcAMP effect on cell morphology was reversible and was completely blocked when the cells were grown in serum free medium containing epidermal growth factor (20 µg/ml). The inhibitory effect of DBcAMP on the cell transformation correlated directly with a decrease in the levels of the 21,000-dalton transforming protein (p21) of the v-ras^H gene as revealed by immunoprecipitation and Western blotting analysis of p21. Moreover, the decrease in p21 levels following DBcAMP treatment was inversely correlated with an increase in the levels of the 54-56K M.W. R^{II} cAMP receptor protein in the cells. These results suggest that cAMP may be an intracellular regulator of ras oncogene.

Two classes of cAMP analogs which show synergism of binding to type II protein kinase synergistically inhibit v-ras^H oncogene expression: cAMP in mammalian cells functions by binding to the cAMP receptor proteins, the regulatory subunits (R^I, R^{II}) of cAMP-dependent protein kinases type I and type II. The regulatory subunit of protein kinase has two different cAMP-binding sites, sites 1 and 2. Two classes of cAMP analogs specific for the site 1 (c-8 analogs) and site 2 (c-6 analogs) demonstrate synergism in combination and this synergism becomes optimal for either type I or type II protein kinase depending on the atom attached on the c-8 position. We studied the regulatory role of cAMP in the expression of the v-ras^H oncogene utilizing these site-specific cAMP analogs. Clone 13-3B-4, a Ha-MuSV DNa transfectant of NIH-3T3 cells, grown in a serum free defined medium was used in the present study. Treatment of cells with a c-6 analog, N^6, $O^{2'}$-dibutyryl cAMP (DBcAMP) (10^{-3}), or with c-8 analogs, 8-Br-cAMP (10^{-4}M) or 8-thiomethyl cAMP (10^{-5}M) for 24 hr resulted in the decrease of p21 ras protein synthesis to 15% of that in untreated cells. The treatments in combination of DBcAMP (10^{-4}M) with either 8-Br-cAMP (10^{-5}M) or 8-thiomethyl cAMP (10^{-6}M) synergistically inhibited p21 synthesis, indicating a type II protein kinase response. In fact, the decrease in p21 levels in 13-3B-4 cells correlated with a change in the molecular species of cAMP receptor proteins (R^I, R^{II}). In the cells treated with cAMP analogs, the concentration of R^{II} tripled while R^I content declined to one-fifth of the untreated cell level, resulting in the ratio of R^I/R^{II} closely resembling that of untransformed 3T3 cells. Indirect immunofluorescence revealed that within 30 min following treatment with cAMP analogs, a high intensity of immunofluorescence of R^{II} appears in nuclei of 13-3B-4 cells. A high intensity of immunofluorescence of R^{II} was also observed in the nuclei of untransformed NIH-3T3 cells, whereas immunofluorescence of R^{II} was undetectable in the transformed 13-3B-4 cells. These changes in the levels of p21 and cAMP receptor proteins in 13-3B-4 cells following cAMP treatment correlated with a change in cell morphology. Cells treated with cAMP analogs exhibited a morphology characteristic of untransformed fibroblasts, while the untreated cells retained a transformed phenotype. These results suggest a role for cAMP and its receptor protein, R^{II}, in the quantitative modulation of v-ras^H oncogene expression.

That cyclic AMP inhibits the expression of the viral ras^H gene of cultured fibroblasts having its own promoter or the MMTV promoter, and of the cellular ras^H gene of mammary carcinomas in vivo, suggests the action of cyclic AMP

at a regulatory locus that may be present in both cellular and viral ras genes. Cyclic AMP in this action may involve its receptor proteins (binding proteins): the role of cyclic AMP + receptor complex at the nuclear level has been suggested to be essential in the cyclic AMP-induced regression of mammary carcinomas.

These in vitro systems may serve as a model to study the precise role of cyclic AMP on the ras gene expression and would eventually help to understand the regulation of cellular proto-oncogene expression in vivo.

Significance to Biomedical Research and the Program of the Institute:

This study contributes to the understanding of the mechanisms of reverse transformation. The antagnostic action between a steroid hormone and cAMP on a hormone-responsive promoter that regulates the oncogenic expression may be of great importance in the understanding of cancer etiology; thus extension of this knowledge will contribute to prevention and better treatment of cancer.

Proposed Course of Research:

To extend the investigation on the mechanism of oncogene expression, the following proposal is made:

1. Assess the relationship between the effect of cAMP on phenotypic reversion of transformant and expression of the transforming ras gene transcript and its product p21.

2. Assess whether the antagonism between glucocorticoid receptor and cAMP receptor is exerted directly on MMTV-LTR.

3. Assess intracellular localization of glucocorticoid- and cAMP-receptor by immunocytochemical method.

4. Assess whether other MMTV-LTR linked gene expression is also affected by cAMP.

5. Assess effect of cAMP on p21 expression directed by other viral or cellular promoter.

6. Assess the effect of R^{II} cAMP receptor microinjected into the Ha-MuSV or MoLV-LTR-c-ras^{H} transformed cells.

Publications:

Cho-Chung, Y.S., Huang, F.L. and Kapoor, C.L.: Role of cyclic AMP in modifying the growth of mammary carcinomas: genomic regulation. In Mihich, E. (Ed.): Biological Responses in Cancer: Progress toward Potential Applications. New York, Plenum Publishing Corp., (In press).

Cho-Chung, Y.S., Clair, T., DeBortoli, M.E., and Tagliaferri, P.: Suppression of cellular and viral ras oncogene expression by cyclic AMP. In Neiburgs, H.E. (Ed.).: Cancer Detection and Prevention. New York, Alan R. Liss, Inc., (In press, 1985).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Enhancement of Oncogene Expression and Mammary Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Y.S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBD, NCI
M. DeBortoli	Visiting Associate	LTIB, DCBD, NCI
P. Tagliaferri	Visiting Fellow	LTIB, DCBD, NCI
T. Clair	Chemist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)* F. L. Huang, LCCTP, NCI
Dr. W. R. Miller, U. of Edinburgh, Scotland
Dr. B. E. Haley, U. of Wyoming, Dept. of Biochemistry, Laramie, Wyoming
Dr. H. Abou-Issa, Ohio State U., Dept. of Surgery, Columbus, Ohio

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SECTION
Cellular Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.2	2.5	0.7

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☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Over twenty distinct transforming genes have been identified in the genomes of oncogenic retroviruses. Each of these oncogenes has a homologue in the chromosomal DNA of a vertebrate species. Current evidence indicates that this highly conserved set of genes may play a vital role in cell proliferation and/or differentiation. In addition, inappropriate expression of some of these genes has been implicated in the genesis of cancer. Our hypothesis is that deregulation of oncogene expression may be a possible general mechanism for the induction of neoplasia in humans. Our efforts have been concentrated on the cellular homologue of the ras gene, the oncogene carried by Harvey and Kirsten Sarcoma viruses. In this study we are investigating the role of ras gene expression in the induction of rat and human mammary carcinomas. In a study of more than 200 human breast carcinomas. We have observed elevated expression of c-ras^H in 70% of estrogen and progesterone receptor positive tumors and 40% of estrogen and progesterone receptor negative tumors. Whereas, an amplified or rearranged c-ras^H gene has not been detected in human mammary carcinomas. Thus, the mechanism by which c-ras^H gene expression is deregulated in these mammary tumors remain to be determined. To study the mechanism of the enhanced c-ras^H gene expression, we will determine the c-ras^H expression in growing and growth-arrested human breast cancer cells (MCF-7), growing vs regressing rat mammary tumors, hormone-dependent vs hormone-independent tumors, and the mammary gland of rodents during normal development and chemical or viral carcinogenesis. Moreover, using a new acceptor cell line, the mouse mammary epithelial cell line (NMuMG) for transfection experiments, we will investigate the intracellular factors those regulate the expression of c-ras^H gene. The goal of this proposal is to provide us a fundamental basis for better understanding the mechanisms by which oncogenes involved in the neoplastic development and growth.

Project Description

Objectives:

To investigate the relationship between hormone-dependent growth of mammary carcinoma and expression of c-ras^H oncogene. To elucidate the mechanism for deregulation of c-ras^H expression, the effect of steroid hormones on the ras^H gene expression will be determined in mammary tumors in vivo and the ras gene transfected culture mammary epithelial cells in vitro.

Methods Employed:

1. Tumors: Primary and metastatic human breast tumors and benign beast diseases, and primary, 7-12-dimethylbenz(α)anthracene (DMBA)-induced rat mammary carcinoma and transplantable MTW9, MTW9A, DMBA #1, MT13762 mammary carcinomas in rats were used. Tumor regression was produced by hormone-withdrawal or DBcAMP (10 mg/day/200 g rat s.c.) treatment. Primary and metastatic, estrogen and progesterone defined human breast carcinomas as well as benign tumors and normal breast tissues were obtained from the Ohio State University, Breast Cancer Research Laboratory, Columbus, Ohio and the University of Edinburgh, Edinburgh, Scotland.

2. MCF-7 cells: The MCF-7 cells (Mason Reserach Institute) were grown in McCoy's 5A medium supplemented with bovine insulin, penicillin, streptomycin and fetal calf serum (1040) and ± additives.

3. NMuMG cells: The NMuMG cells (normal mouse mammary epithelial cells) were grown in DMEM containing penicillin, streptomycin and supplemented with 10% fetal calf serum. The cells were transfected with Ki-ras-neo DNA or DNA from DMBA-induced mammary tumors.

4. mRNA: Total polyA containing mRNA was isolated from tumors by the method of Deeley et al. In vitro translation systems of both rabbit reticulocyte lysate and wheat germ extract were used. Total translation products were analyzed by SDS-PAGE.

5. p21 Immunoprecipitation: [^{35}S] methionine-labeled tumor cell lysates or the in vitro translation products of tumor mRNAs were incubated for overnight at 4°C with rat monoclonal antibody against Harvey sarcoma virus p21. The reaction mixture was then incubated for an additional 2.0 hr at 4°C with a 10% (vol/vol) suspension for Formalin-fixed Staphylococcus aureus (precoated with rabbit anti-rat immunoglobulin G), then immunoprecipitates were collected by centrifugation, washed and analyzed by electrophoresis in SDS/polyacrylamide gels.

6. Immunoblotting of p21 ras protein: Cellular proteins present in the tumor extracts were separated by 12% $NaDodSO_4$-PAGE and transferred to nitrocellulose. Nitrocellulose sheets were first incubated with 3% bovine serum albumin solution (37°C, 3hr) to remove bound $NaDodSO_4$ and to block the residual sites on the cellulose, then sequentially exposed to p21 monoclonal antibody #Y13-259, directed against the Harvey sarcoma virus-encoded p21 rabbit anti-rat IgG, and

^{125}I-labeled protein A and were finally exposed for autoradiography.

7. 8-N_3-γ-[^{32}P]GTP-binding of p21: For photo-activated 8-N_3-α-[^{32}P]GTP incorporation, tumor lysates were incubated in dark with 5 x 10^{-6}M 8-N_3-γ-[^{32}P]GTP (35 ci/mmol) for 90 min at 23° with vigorous shaking, exposed to UV light for 30 sec, quenched, and subjected to SDS-PAGE.

8. Molecular clones of viral DNAs: Molecular clones containing the transforming sequences of Harvey sarcoma virus (ras^H) and Kirsten sarcoma virus (ras^K) in plasmid pBR322 were used as probes to detect cellular ras transcripts in tumors. BS-9 contains a 0.5 kilobase (kb) fragment of ras^H and HiHi-3 contains a 1.0-kb fragment of ras^K sequences. Plasmid DNAs were ^{32}P-labeled by nick-translation to specific activities of approximately 4 x 10^8 cpm/μg DNA for use as hybridization probes.

9. Blot hybridization analysis: Procedures for digestion of cellular DNAs with restriction endonucleases, electrophoresis in agarose gels, and transfer to nitrocellulase filters followed the method described in (Cell 19: 863, 1980). Prehybridization, hybridization, and washing procedures were as described by Hanahan and Meselson (Gene 10: 63, 1980) except that 10% dextran sulfate was included in the hybridization buffer (Proc. Natl. Acad. Sci. USA 76: 3683, 1979).

Major Findings:

Enhanced expression of c-ras^H oncogene in hormone-dependent mammary carcinomas in rats: The in vitro translated proteins from poly(A)+ RNAs differed when hormone-dependent rat mammary carcinomas were compared during their growth and regression (Huang and Cho-Chung, Cancer Res. 43: 2138, 1983). In this study, we present evidence that the 22K M.W. protein which shown an amplified translation in the growing tumor as opposed to the regressing tumor, represents a transforming gene product associated with an oncogenic expression. A monoclonal antibody, #Y13-259, that reacts with the 21K transforming protein (p21) encoded by the v-ras gene of Harvey murine sarcoma virus (Ha-MuSV) specifically immunoprecipitated the 22K translation product of the growing tumors. In contrast, an insignificant amount of p21 was detected in the translation products from the regressing tumors following either hormone-withdrawal (ovariectomy) or dibutyryl cyclic AMP treatment. The monoclonal antibody, #Y13-238, that efficiently binds only the Ha-ras-p21 species but not Ki-ras p21 also immunoprecipated the 22K translation protein suggested that the tumor p21 is the product of a Ha-ras gene. These results suggest that enhancement and suppression of a cellular ras oncogene is associated with the growth and regression of mammary tumors, respectively.

p21 expression in growing and regressing tumors, and in normal mammary glands of female virgin Sprague-Dawley rats, was estimated quantitatively. The concentrations of the immunoprecipitated p21 protein were compared from densitometric tracings of the band intensities from the autoradiograms of SDS-polyacrylmaide gels. It was found that the levels of p21 in the translation products of the regressing tumors at day 3 after ovariectomy or dibutyryl cyclic AMP treatment were only 15% of those found in the translation products of growing tumors.

It was also found that the levels of p21 in the translation products of the growing tumor were tenfold greater than those found in the mammary glands, suggesting that the growing tumors may contain an increased amount of p21 mRNA.

The change in translated p21 level observed during growth and regression appears to be specifically related to the hormone-dependency of mammary tumors. Approximately 12% of DMBA-induced mammary tumors that have failed to regress and continued to grow after ovariectomy or dibutyryl cyclic AMP treatment contained the levels of translated p21 as low as that in virgin mammary glands and the p21 levels did not change after either ovariectomy or dibutyryl cyclic AMP treatment.

Western blotting analysis was used to determine the amounts of p21 ras protein in the mammary tumors. The levels of p21 in hormone-dependent DMBA-induced tumors were about 7-fold that of hormone-independent tumors (DMBA#1 and NMU-induced). The hormone-independent mammary tumors exhibited a low level of p21 as those of normal mammary glands.

This study presents the first evidence that the in vivo growth of a primary hormone-dependent mammary carcinomas is associated with enhanced expression of a cellular oncogene, c-ras^{H}. That hormone-withdrawal induces decrease in the c-ras^{H} gene expression in the regressing tumors suggests a possible causal role for this oncogene in the hormone-dependent growth of mammary tumors. Infact, the p21 expression in the hormone-dependent mammary tumors is tenfold higher than that in the virgin mammary gland, whereas in hormone-independent mammary tumors, the expression of p21 was not elevated. It is probable that hormone-stimulated enhancement of c-ras^{H} expression might be correlated with hormone-dependent growth of mammary cancer.

Elevated levels of p21 ras protein in human breast carcinomas: Western blotting analysis of p21 in over 200 primary human breast carcinomas revealed that the majority (68%) of estrogen and progesterone receptor positive breast tumors contained a high level of p21. The p21 levels in these receptor positive tumors were 10-fold that of the normal breast tissue. A fraction (32%) of the receptor negative breast tumors, however, also contained a high level of p21 protein. These receptor negative tumors exhibiting a high level of p21 were the advanced breast cancers. In the early breast cancers, none of the receptor negative tumors contained high levels of p21. Thus, a correlation between elevation of p21 and hormone-dependence of mammary tumors was found in early breast cancers. The results suggest that the estimation of p21 levels in early breast cancers may be a good predictive and prognostic value of the tumors.

Photo-incorporation of 8-N_3-[γ ^{32}P]GTP into mammary tumor lysates: GTP or (GDP)-bindng is the only known biological function common to both v-ras p21 and c-ras p21. We examined whether the p21 species identified in the human and rat mammary tumors would exhibit guanine nucleotide binding activity by the use of the photoaffinity ligand, 8-N_3-[γ-^{32}P]GTP. Tumor lysates containing either high or low levels of p21 were incubated with 8-N_3-[γ-^{32}P]GTP for various lengths of time. The incubation mixtures were then exposed to UV light, quenched, and subjected to NaDodSO$_4$-PAGE. Only in these lysates containing low levels of p21, was there a time-dependent appearance of a GTP-labeled protein

band of MW 21K, presumably p21, as well as other labeled protein bands. These protein bands were present only after photolysis with UV light, indiciating a covalent attachment of the labeled nucleotide to the proteins. That the tumor lysates containing high levels of p21 did not exhibit labeling of p21 as well as other GTP binding proteins suggested the presence of high GTPase activities in the lysates. In fact, 8-N_3-[γ-^{32}P]GTP photo-incorporation into mixtures of lysates containing igh and low levels of p21 resulted in a complete loss of the p21 band and marked decrease of other GTP binding protein bands. The low photo-incorporation of 8-N_3-[γ-^{32}P]GTP into the tumor lysates containing high levels of p21 was not due to low GTP binding activity: direct binding of [α^{32}P]GTP or [γ-^{32}P]GTP to p21 transferred to nitrocellulose sheets was found to be proportional to the amount of p21 present in the tumors. Thus, the elevated p21 in the mammary carcinomas was accompanied by a high GTPase activity. In the light of a recent report that 'normal' p21 possesses GTPase activity, while in mutant p21 this function of GTPase is impaired, the elevated p21 in the hormone-dependent mammary carcinomas may be 'normal' p21 rather than structurally altered protein. Thus, amplified, hormone-stimulated expression of 'normal' p21 may be involved in the hormone-dependent growth of mammary carcinomas. Thus, Western blotting analysis and photoactivated binding of 8-N_3-[γ-^{32}P]GTP now make possible both quantitative and qualitative assessments of p21 expression in tissues.

Significance to Biomedical Research and the Program of the Institute:

This study contributes to the understanding of the mechanism by which oncogenes involved in the neoplastic development and growth. The finding that enhancement and suppression of cellular ras oncogene expression correlate with growth and regression of mammary tumors, respectively, is consistent with the hypothesis that ras gene expression plays an important role in oncogenesis of mammary tumors. Moreover, cAMP and steroid hormone-interaction may play an important role in oncogenesis since treatment with either dibutyryl cyclic AMP treatment or hormone withdrawal (ovariectomy) resulted in tumor regression as well as suppression of the ras gene expression. Finally quantitative determination of ras gene expression may contribute to the early diagnosis of mammary cancer.

Proposed Course of Research:

To extend the investigation of the mechanism of oncogenesis of mammary tumors, the following proposal is made:

1. Assess the relationship between mammary tumorigenesis and expression of ras gene at the level of both ras transcript and ras gene product, p21.

2. Assess whether levels of p21 in human breast cancers measured by GTP-binding and immunoblotting can be a predictive value for hormone-dependency and prognosis of mammary tumors.

3. Assess the role of steroid hormones and cAMP in modulation of ras gene expression during mammary gland development and mammary carcinogenesis.

4. Assess whether DNA of DMBA mammary carcinomas can transfect NMuMG cells (normal mouse mammary epithelial cells) and the transforming gene of tumor is homologous to the ras gene of Harvey sarcoma virus.

5. Assess hormone-stimulation of c-ras^H expression in the c-ras^H DNA transfected NMuMG cells growing in culture and in nude mice in vivo.

Pubications:

Huang, F.L. and Cho-Chung, Y.S.: Hormone-regulated expression of cellular ras^H oncogene in mammary carcinomas in rats. Biochem. Biophys. Res. Commun. 123: 141-147, 1984.

DeBortoli, M.E., Abou-Issa, H., Haley, B.E., and Cho-Chung, Y.S.: Amplified expression of p21 ras protein in hormone-dependent mammary carcinomas of humans and rodents. Biochem. Biophys. Res. Commun. 127: 699-706, 1984.

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Cho-Chung, Y.S., Huang, F.L., and Kapoor, C.L.: Role of cyclic AMP in modifying the growth of mammary carcinomas: genomic regulation. In Mihich, E. (Ed.): Biological Responses in Cancer: Progress toward Potential Applications. New York, Plenum Publishing Corp., (In press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08249-06 LTIB
formerly
Z01 CB 08249-05 LPP

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Hormonal Control of Growth of Normal and Neoplastic Mammary Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

William R. Kidwell	Chief, Cell Cycle Regulation Sect.	LTIB, DCBD, NCI
David Salomon	Supv. Res. Biologist	LTIB, DCBD, NCI
Sanjeeva Mohanam	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Growth factors that stimulate the proliferation of normal and neoplastic mammary cells have been purified from rodent and human breast tumors and from human milk. The factors from the latter two sources have been purified to apparent homogeneity and are probably identical. This factor (MDGF1) exerts its biological effects via specific, high affinity, membrane receptors. However, evidence for a synergism with estrogen for proliferation has been obtained. The mammary cell's ability to respond to MDGF1 is dependent on the substratum on which the cells are grown. Stromal collagen and fibronectin potentiate the response while laminin and basement membrane collagen inhibit it. These effects may be due to modulation of MDGF1 receptors. EGF and MDGF1 responsiveness of mammary cells are similarly affected by the culture substratum. EGF receptor number and affinities are the same on all four substrata when cells are grown in the absence of EGF. However, in the presence of a receptor down-regulating level of EGF, the cells regenerate membrane receptors more efficiently on stromal collagen and fibronectin than on type IV collagen or laminin. This difference in receptor number (2-3 fold) may explain the differing responsiveness to growth factors on the various substrata. It could be physiologically relevant since the epithelium is believed to penetrate the pre-existing basement membrane and contact stromal elements as it invades the surrounding tissue in response to a proliferative stimulus. Maintenance of a sufficient number of growth factor receptors could assure that the cells are competent to regenerate a new basement membrane since both EGF and MDGF1 greatly potentiate the cells ability to make new collagen via elevating collagen mRNA levels in cells.

GPO 914-018

Project Description

Objectives:

Previous studies have suggested that production of basement membrane proteins is important for the growth of normal mammary epithelium and adenocarcinomas derived from it. The present studies are aimed at elucidating the mechanism whereby production of this extracellular matrix is regulated. A growth factor which differentially stimulates matrix production has been purified from human mammary tumors. Our objective is to determine whether this is an autocrine-like growth factor and to define the mechanism by which its effects on mammary cells are produced.

Methods Employed:

Collagen synthesis stimulating activities have been extracted from rat, mouse and human mammary tumors with acidified ethanol. Factor purification included gel filtration, ion exchange column chromatography, isoelectric focusing gel electrophoresis and high performance liquid chromatography. Effects on collagen synthesis were assessed by quantitating the relative amount of ^{14}C-proline incorporated into collagenase sensitive protein in mammary cell cultures or alternatively measuring mass of 4-hydroxyproline, 3-hydroxyproline and hydroxlysine formed by the cell cultures. cDNAs against collagen I were used in RNA and cytoblot hybridizations to quantitate collagen mRNA levels in MDGF1 treated NRK cells using standard techniques.

Major Findings:

1. Myoepithelial or basal cells have been shown to be the major collagen IV synthesizing cell type in both normal rat mammary epithelium and in mammary adenocarcinomas derived from it. These cells have been isolated in high purity form rat mammary adenocarcinomas. In culture these cells synthesize 50-75 times as much collagen as the epithelial cells (Cancer Res. 44: 3055, 1984). Rat mammary tumors that contain this cell population make basement membranes. The cell type is absent from carcinomas which do not make a basement membrane. The former tumors also contain a growth factor that potentiates collagen IV synthesis in normal mammary cell cultures. The carcinoams possess little or none of this factor (Progress in Cancer Researach and Cancer, Vol. 31, p. 129, 1984). These results suggest that the growth factors present in the adenocarcinomas are autocrine factors that regulate basement membrane synthesis, presumably by their action on the myoepithelial cell population of the tumor and normal mammary epithelium.

2. A growth factor that biologically resembles that present in rat mammary adenocarcinomas has been purified to apparent homogeneity from human breast tumors and human milk. This factor is a 62,000 Mr protein that is pepsin sensitive, heat stable, S-S reducing agent insensitive and has an acidic pI. The factor interacts with high affinity membrane receptors on mammary cells (Kd = 10 pMolar) and potentiates the cells to differentially amplify their production of basement membrane collagen and laminin. This effect is correlated with the elevation of collagen mRNA in the cells. The growth factor, named MDGF1, does not stimulate mammary cell collagen production if

the cells are plated on basement membrane collagen. These results suggest that MDGF1 acts to facilitate new basement membrane synthesis in adenocarcinomas and normal mammary cells as they move from their resting position on basement membranes and invade the stroma during proliferation (J. Biol. Chem. 260: 5745, 1985).

3. Suppression of the MDGF1 responsiveness of mammary cells by basement membrane collagen appears to be related to the effects of this matrix component on growth factor receptors. EGF was found to produce a similar biological effect on mammary cells as does MDGF1, with EGF responsiveness also being suppressed by basement membrane collagen (type IV) compared to stromal collagen (type I). EGF receptors were quantitated on normal mammary cells growing on type I or type IV collagen, laminin or fibronectin coated dishes. In the absence of EGF the cells expressed the same number of receptors with the same affinity constants. When EGF was added, the EGF receptors were down-regulated with similar kinetics. However, on the stromal collagen and fibronectin there was a rebound of receptors that did not occur on type IV collagen or laminin. This is precisely what one would predict if basement membrane components negatively feed-back regulated their own biosynthesis. These observations indicate that it is not just the levels of growth factors accessible to cells that determine their growth response, but also the physical environment in which the cells find themselves. Is this pertinent to the growth regulation of metastatic mammary tumor cells that do not deposit a basement membrane?

4. In addition to the negative-feedback and growth factor production aspects, basement membrane synthesis appears to be controlled by the conversion of mammary stem cells into myoepithelial cells. We have found that certain mouse mammary cell lines plated on or within collagen I gels generate new cell types that are believed to be myoepithelial cells. A similar cell type is also seen when these cells are transplanted into immuno-incompetent mice. This may provide us with a good model system for determining how tumors progress from a state in which they are dependent on basement membrane biosynthesis (adenocarcinomas) to a state in which this matrix component is no longer required (carcinomas).

Significance to Biomedical Research and the Program of the Institute:

We have demonstrated that blocking collagen synthesis leads to the growth arrest of mammary adenocarcinomas. Further studies of collagen synthesis controls may provide a variety of methods by which tumor growth arrest can be accomplished in the breast cancer patient. These studies should provide fundamental information about the role of collagen in the growth and differentiation of normal breast epithelium and the significance of collagen production by neoplastic cells derived from it.

Proposed Course for Research:

Our objective in preparing monoclonal antibodies against MDGF1 have thus far not been realized. We will attempt to generate polyclonal antibody by the

intrasplenic injection method of Sell. If we are successful, these antibodies will be utilized to determine if MDGF1 is indeed synthesized by human breast cancer cells and if so, whether the growth factor production is modulated by pituitary hormones and/or ovarian steroids. Additional amounts of MDGF1 will be prepared for structural analysis and for characterization of MDGF1 receptors on breast tumor cells. If evidence for the production of MDGF1 is obtained, we will attempt to clone the factor using synthetically prepared oligodeoxynucleotides corresponding to the amino acid sequence of the factor by screening a cDNA library that we have prepared against MCF-7 cells or by screening a genomic library of a human tumor cell line. Our studies of substratum effects on growth factor receptor expression has, to date, been limited to EGF because of a lack of sufficient amounts of MDGF1. With additional supplies of the growth factor in hand, we will examine the possibility that its receptor, like that for EGF, is differentially modulated by contact of mammary cells with basement membrane components.

Publications:

Bano, M., Lewko, W.L., and Kidwell, W.R.: Characterization of rat mammary tumor cell populations. Cancer Res. 44: 3055-3062, 1984.

Kidwell, W.R. and Shaffer, J.: Growth stimulatory effects of unsaturated fatty acids for normal and neoplastic breast epithelium. J. Amer. Oil Chemists' Soc. 61: 1900-1904, 1985.

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Kidwell, W.R., Bano, M. and Salomon, D.S.: Purification of growth factors from human milk. Amer. J. Clin. Nutr. (In press).

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Zwiebel, J.A., Bano, M., Nexo, E., Salomon, D.S. and Kidwell, W.R.: Partial purification of transforming growth factors from human milk. Cancer Res. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08279-04 LTIB
formerly
Z01 CB 08279-03 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effect of Proline Analogs on Normal and Neoplastic Breast Epithelium

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

W. R. Kidwell	Chief, Cell Cycle Regulation Sec.	LTIB, DCBD, NCI
F. Grantham	Bio. Lab. Tech.	LTIB, DCBD, NCI
S. Liu	Bio. Lab. Tech.	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

M. Sobel, Laboratory of Pathology, National Cancer Institute, NIH

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cell Cycle Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.2	0.8

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

D

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A series of proline analogs have been analyzed for their effects on collagen synthesis inhibition in cultures of primary DMBA-induced rat mammary tumors and for their effects on mammary tumor growth in tumor bearing animals. Azetidine carboxylate, thioproline and cis-hydroxyproline were found to be potent, selective inhibitors of collagen synthesis, blocking amino acid incorporation into collagen by 7 to 27 fold more than incorporation into total tumor cell protein. In vivo all 3 of these compounds at doses of 50-200 mg/kg S.C., caused tumor growth arrest or regression. The conditions favoring proline analog sensitivity of mammary tumors have been assessed. A positive correlation exists between the ability of a tumor to synthesize basement membrane and its proline analog sensitivity. Sensitivity is approximately proportional to the efficacy of the analog in blocking collagen synthesis in cultured tumor epithelium. The epithelium of normal mammary glands and mammary adenocarcinomas is dependent on proline for optimal growth, especially when cells are plated on stromal collagen substrata. Blocking basement membrane deposition and thereby favoring tumor cell contact with stroma may, therefore, promote proline analog uptake and tumor cell kill. In contrast to primary tumors, metastatic rat mammary tumor growth was not affected by proline analogs. Electron microscopy revealed that the latter tumors lacked a basement membrane. Using cDNAs against type IV collagen, we have demonstrated a lack of collagen IV mRNA sequences in the metastatic tumors whereas the collagen message is present in collage IV producing, proline analog sensitive, primary tumors.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

Basement membrane collagen synthesis appears to be important for the growth of normal mammary epithelium and for well differentiated mammary tumors as shown by the ability of proline analogs to kill the cells from either source in vivo or in vitro. This is in contrast to poorly differentiated, metastatic mammary tumors whose growth is unaffected by the proline analogs. The latter tumors either do not produce a basement membrane or such structures, if produced, are degraded by the tumor. This possibility has been examined by quantitating the levels of RNA sequences complementary to type IV collagen message in metastatic and non-metastatic rat mammary tumors.

Methods Employed:

Two metastatic rat mammary cell lines, one induced by N-methylnitrosourea (NMU) and one by 7,12-dimethylbenz(α)anthracene (DMBA) have been established in culture. The RNA from these cells, and that of primary NMU and DMBA cells has been isolated using isothiocyanate-guanidium-HCl. A cDNA corresponding to type IV collagen with low cross reactivity for other collagen mRNAs obtained from M. Sobel was used to quantitate collagen IV mRNA by slot blot or Northern blot analysis.

Major Findings:

Cultures of the primary rat mammary tumors induced by either NMU or DMBA were found to synthesize collagenase sensitive proteins that electrophoresed with authentic type IV collagen on denaturing acrylamide gels run under reducing conditions. In contrast, no detectable type IV collagen was made by the metastatic mammary tumor cell lines. These results were consistent with previous studies showing a lack of staining of the metastatic tumors with anti type IV collagen antibodies. When RNA was prepared from the primary and metastatic tumors and hybridized under stringent conditions to cDNA for type IV collagen (alpha 1 chain), the primary, non-metastatic tumors were found to possess about 4-10 times as much type IV collagen related RNA sequences than the metastatic cells. When the tumor cell RNAs were electrophoresed and analyzed by the Northern technique, the primary tumors were found to contain a 3 kb RNA that hybridized under stringent conditions to the collagen IV cDNA. This RNA species was totally absent from the metastatic tumor cell lines. The published size of type IV collagen mRNA is about twice twice that of the putative type IV collagen mRNA we have detected from the rat mammary tumors. Calculations indicate that a 3 kb mRNA could encode a protein of about 120,000 Mr, or 2/3 the size of procollagen IV. These results suggest that the primary tumors produce a collagen IV mRNA as well as second closely related mRNA. Alternatively, they suggest that collagen IV mRNA is turning over rapidly and fragments of the message are accumulating in the non-metastatic tumors but not in the metastatic tumors.

Significance to Biomedical Research and the Program of the Institute:

The production of a basal lamina appears to be essential for the growth of normal mammary epithelium and differentiated tumors arising from it. However,

by some mechanism tumors are capable of progressing to a state in which this requirement is lost. Such a state is metastasis. Current evidence suggests that this latter state is characterized by the loss of the myoepithelial cell component, the cells which we have shown to be primarily responsible for the production of the basal lamina. Our recent findings suggest that the metastatic cells fail to produce a basal lamina in part because they have lost the ability to produce mRNAs for lamina proteins. Thus, the lack of such mRNA species may be a marker for the development of metastatic potential.

Proposed Course of Research:

The available evidence suggests that basal lamina production is regulated by the conversion of stem cells to myoepithelial cell types and that these latter cells synthesize most of the lamina components, especially type IV collagen. To understand why metastatic mammary tumor cells have lost the ability for net production of the lamina, we have developed normal mammary cell lines which can be greatly stimulated to differentiate into myoepithelial-like cells under the appropriate culture conditions. The mechanisms by which the stem cell conversion takes place will be evaluated by quantitating both the levels of lamina proteins and their mRNAs. Additionally, we will test our hypothesis that the 3 kb RNA species is a marker for metastatic potential by quantitating the levels of the RNA in metastatic and non-metastatic human breast tumors. The 3 kb RNA from the rat mammary tumors will be isolated, cloned and sequenced to determine if it is a fragment of collagen IV mRNA or a collagen IV-related sequence.

Publications:

Bano, M., Lewko, W., and Kidwell, W.R.: Characterization of rat mammary tumor cell populations. Cancer Res. 44: 3055, 1984.

Kidwell, W.R., Taylor, S., Bano, M., and Grantham, F.: Growth arrest of mammary tumors by proline analogs. In Bresciani, F., King, R., Lippman, M., Namer, M. and Raynaud, J-P. (Eds.): Hormones and Cancer, Vol. 31, Raven Press, New York, 1984, pp 129-133.

Kidwell, W.R., Smith, G.S. and Vonderhaar, B.K.: Substratum modulation of growth factor responsiveness of mammary epithelial cells. In Mather, J., Barnes, D., and Sato, G. (Eds.): Growth and Differentiation of Cells in Defined Environments, Fukuoka Conference Symposium. In press.

Kidwell, W.R., Bano, M. and Taylor, S.: Mammary tumor growth arrest by collagen synthesis inhibitors. In Mihich, E. (Ed.): Novel Cancer Chemotherapeutic Agents, Academic Press, Inc., New York. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05211-13 LTIB
formerly
Z01 CB 05211-12 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Poly(ADP-ribose) and Chromatin Structure and Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

W. R. Kidwell	Chief, Cell Cycle Reg. Sec.	LTIB, DCBD, NCI
M.R. Purnell	Visiting Fellow	LPP, DCBD, NCI

COOPERATING UNITS *(if any)*

Joel Moss, Sr. Staff Scientist, HIR, CM

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cell Cycle Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Poly(ADP-ribose) synthetase is a chromatin bound enzyme that adds chains of ADP-ribose in tandem to nuclear proteins. This enzyme is activated by DNA damaging agents such as gamma, x-ray and u.v. irradiation and by DNA alkylating agents. We have synthesized and tested 6 compounds which are inhibitors of the synthetase and found that the ability of 4 of 6 of them to block DNA repair is directly correlated with the compound's potency as a synthetase inhibitor. The compounds ranked in order of their ability to block DNA repair are 3-acetl-aminobenzamide> 3-hydroxybenzamide= benzamide>>3-aminobenzamide. 3-nitrobenzamide, was found to be much more inhibitory for the repair of DNA chain breaks than was expected based on its potency as a poly(ADP-ribose) synthetase inhibitor. Plots of the reciprocal of the repair velocity vs inhibitor concentration normalized against its k_i for synthetase were made. These plots were biphasic indicating that the benzamides had effects on more than one cellular process. At least two processes other than DNA repair have been implicated as targets. These are RNA synthesis and glutamine synthetase. The latter enzyme was found to be an acceptor protein for mono-ADP-ribose. The enzyme catalyzing this reaction was also found to be inhibited by the benzamides, though their potency as inhibitors for this enzyme was much less than their potency as synthetase inhibitors.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

A series of benzamide analogs which are variably capable of blocking poly (ADP-ribose) synthetase activity are being evaluated for their effects on cell growth, cell cycle arrest and for effects on DNA repair processes. These experiments are designed to elucidate the role of poly(ADP-ribose) in cell function.

Major Findings:

Poly(ADP-ribose) synthetase inhibitors block DNA repair. A variety of benzamides substituted in the 3'-position were synthesized and their potency as synthetase inhibitors tested. These were 3-acetylaminobenzamide, k_i = 0.43 μmolar; 3-methoxybenzamide, k_i = 0.61 μmolar; benzamide=3-hydroxybenzamide, k_i = 1.0 μmolar; 3-amino-benzamide, k_i =2.8 μmolar; 3-nitrobenzamide, 9.8 μmolar. These compounds were tested for their ability to block the repair of γ-ray induced DNA chain breaks by measuring the effect of the compounds at various concentrations on the single strand break repair rate as determined from alkaline elution of DNA through polycarbonate filters. First order rate constants for the elution of DNA were determined and these were converted into RAD equivalents of DNA breaks. Plots of the reciprocal of the repair rate vs the concentration of the synthetase inhibitor normalized against its k_i were constructed. These results showed that at low inhibitor concentrations the ability of all the benzamides (except 3-nitrobenzamide and 3-methoxybenzamide) to block DNA repair was directly correlated with the potency of the compound as a synthetase inhibitor. Poly(ADP-ribose) synthetase inhibitors block cell division and reduce cell viability. Cytotoxicity of the benzamide analogs were assessed by treating cells for two cell doubling times with various concentrations of the compounds followed by cloning efficiency vs inhibitor concentration normalized against its k_i were constructed. The results indicated that the benzamide analogs caused a loss of cell viability. At all inhibitor concentrations, the benzamides apparently produce these effects on cell viability by blocking poly(ADP-ribose) synthetase. An evaluation of the cytostatic potential of the compounds indicated that cell division could proceed in the absence of poly(ADP-ribose) synthesis but that cumulative damage to cells leading to cell death resulted after 2 cell doublings. One compound, 3-acetylaminobenzamide was studied in detail. It was found to produce a cell growth arrest in G_1 and in G_2. This compound which is the most potent synthetase inhibitor synthesized to date may be very useful for assessing the role of poly(ADP-ribose) in cell cycle progression. Poly(ADP-ribose) synthetase inhibitors may block DNA repair indirectly rather than via inhibiting synthetase. ^{3}H-uridine uptake into RNA was found to be dramatically and rapidly affected by the synthetase inhibitors. The most potent inhibitor was 3-nitrobenzamide. Plots were made of the electron withdrawing potency of the 3-substituents of the analogs (Hammet constant) and the % inhibition of RNA synthesis and these demonstrated a linear relationship. A plot of the RNA synthesis rate vs inhibitor concentration normalized against k_i indicated that the effect of the benzamides in blocking RNA synthesis was probably not due to the effect of these compounds on poly(ADP-ribose) synthetase since the curves

obtained with individual inhibitors were not superimposable. Poly(ADP-ribose) synthetase inhibitors synergistically act with DNA damaging agents to kill cells. Although their mechanism of action may be independent of effects on poly(ADP-ribose) synthetase, the benzamides potentiated the killing effects of NMU, bleomycin, methylmethane sulfonate and adriamycin. These effects were seen at concentrations of benzamides which were by themselves not appreciably cytotoxic or cytostatic. The synergistic killing potential was inversely proportional to the k_i of the benzamides except for 3-nitrobenzamide and 3-methoxybenzamide, the former being more potent and the latter less potent than predicted from their k_i for synthetase. A second enzyme, NAD: Arginine ADP-ribosyltransferase, which also catalyzes the transfer of ADP-ribose to acceptor proteins in the cytoplasm was considered as a possible target for the effects of the benzamides on cell viability, DNA repair, and RNA synthesis. In collaboration with Joel Moss this enzyme was shown to be inhibited by benzamides. The rank order of the inhibitor potencies was similar to that for DNA repair but inhibitor potency was much less than for poly(ADP-ribose) synthetase. Using purified ADP-ribosyltransferase and glutamine synthetase, an enzyme with arginine at its active site, we demonstrated that the transferase selectively transfers one ADP-ribose to glutamine synthetase and thereby inactivates it. Thus, a part of the benzamide effects on cells may be explained by their inhibition of NAD:Arginine ADP-ribosyltransferase.

Significance to Biomedical Research and the Program of the Institute:

Evidence has accumulated which suggests that poly(ADP-ribose) synthetase plays some important role in the maintenance of the fidelity of chromatin structure. The enzyme is activated by a variety of agents that cause DNA damage and becomes deactivated when the damage is repaired. This fact has suggested that chemotherapeutic agent action may be potentiated by compounds that block poly(ADP-ribose) synthetase and prevent DNA repair. Indeed, two recent reports from T. Sugrimura's Laboratory support this postulate. The purpose of our studies is to develop selective inhibitors of the synthetase which are innocuous by themselves but which enhance cell killing by chemotherapeutic agents which cause DNA damage.

Proposed Course of Research:

This project has been temporarily halted.

Publications:

Kidwell, W.R. and Purnell, M.R.: Temperature sensitivity of poly(ADP-ribose) synthetase in whole cells. In Sugrimura, T. and Hayaishi, O. (Eds.): Poly (ADP-ribose) Synthetase and DNA Repair, Japan Sci. Soc. Press, Tokyo, 1983, pp. 243-252.

Kidwell, W.R. and Purnell, M.R.: Poly(ADP-ribose) glycohydrolase and phosphodiesterases in the analysis of poly ADP-ribosylated proteins. In Moldove, K. and Wold, F. (Eds): Posttranslational Modifications; Methods in Enzymology, Academic Press, Inc., New York, 106: 505-510, 1984.

Moss, J., Watkins, P.A., Stanley, S.T., Purnell, M.R. and Kidwell, W.R.: Inactivation of glutamine synthetases by an NAD:Arginine ADP-Ribosyltransferase. J. Biol. Chem. 259: 5100-5104, 1984.

Kidwell, W.R., Purnell, M. and Noguchi, P.: Poly (ADP-ribose) synthetase inhibitor effects on cell function. In Jacobson, M. (Ed.): Proc. 7th Int. Symp. on ADP-ribosylation Reactions. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08274-04 LTIB
formerly
Z01 CB 08274-03 LPP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Lactogenic Hormone Receptors in Mammary Tissue

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

B.K. Vonderhaar	Research Chemist	LTIB, DCBD, NCI
Maria Nascimento	Guest Researcher	LTIB, DCBD, NCI
Ratna Biswas	Visiting Fellow	LTIB, DCBD, NCI
Claudio Dati	Visiting Fellow	LTIB, DCBD, NCI
Erika Ginsburg	Biologist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. Kevin Lynch, University of Virginia, Charlottesville, VA
Dr. Jeffrey Bell, Cornell University, Ithaca, NY

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.75	2.50	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project is designed to evaluate the nature of lactogenic hormone receptors and the factors (including other hormones) which affect binding of the hormone to this molecule. Studies include 1) purification of the receptor from human tissue and preparation and characterization of an antibody against it; 2) examination of the nature of the subunits of the receptor, and 3) characterization of the nature of the interaction of Tamoxifen with membrane-bound receptors related to the lactogen receptor.

GPO 914-918

Project Description

Objective:

The purpose of these studies is to investigate the nature of the interaction of lactogenic hormones with their receptors. The stability of the hormone-receptor complex, and the nature of the cryptic sites was investigated, as well as the effects of alterations in membrane lipids and membrane aggregation. Purification of the lactogenic hormone receptors was attempted in order to prepare antibodies for studies on regulation of synthesis of the molecule.

Methods Employed:

C3H/HeN mice were used. Hormones were iodinated by a lactoperoxidase method. Microsomal membranes were prepared from mammary glands (MG) or livers (L), human breast tumors (MCF-7 grown in nude mice), MCF-7 cells and human placentas using established procedures. Specific binding of prolactin (Prl) or human growth hormone (hGH) as well as ^{3}H-Tamoxifen, to membrane bound or detergent solubilized receptors was performed either in the presence of 1% Triton X-100 or 0.5-1.0% of the zwitterionic detergent CHAPS. Plasma membranes and golgi fractions were purified using standard step-sucrose gradient techniques.

Purification of the receptors was performed using an oPrl affinity column and 4M urea or high $MgCl_2$ to strip the receptor from the affinity matrix, or by an immunoaffinity column using a polyclonal anti-receptor antibody raised in rabbits.

Nb2 rat lymphoma cells (obtained from Drs. Gout and Noble) are propagated in Fischer's leukemic cell medium with 0.15 mM β -mercaptoethanol, 10% horse serum and 10% fetal calf serum or various concentrations of lactogenic hormones. Growth of cells is monitored by direct counting using a hemacytometer.

Major Findings:

In order to study the regulation of the lactogenic hormone receptor itself under controlled conditions, we have begun the purification of the prolactin receptor from human tissue. Recently, we isolated a receptor from mouse liver that binds the lactogenic hormones ovine prolactin and human growth hormone. We now have isolated the receptor from human placenta and from solid MCF-7 tumors grown in nude mice using a modification of this procedure. Placenta has a low number of prolactin receptors which localize, both biochemically and histologically, to the chorion. Large amounts of human placenta are readily available from Bethesda Naval Hospital.

Chorion is dissected from term placentas (age less than 24h, stored at 4°C). Microsomes are then prepared. The microsomes are "stripped" of endogenous lactogen bound to receptor by treatment with 4M $MgCl_2$. At other concentrations, less binding activity is recovered. The microsomes were then restored to low Mg^{2+} buffer, and solubilized with 0.5% CHAPS. Initially, about 40 to 50% of the binding activity is recovered in the supernatant. A second solubilization of the membrane pellet with 0.5% CHAPS can result in a total receptor extraction of 70-80% thus increasing our final receptor yield.

By Scatchard analysis the receptors solubilized by the first treatment with CHAPS, the second CHAPS treatment and the receptors present in the residual pellet all have the same affinity for lactogenic hormones.

The combined supernatants from the CHAPS extractions are then incubated with an ovine prolactin affinity matrix. After washing extensively with buffer to release unbound or loosely bound material, the receptors are eluted batchwise using 4M urea and 1M NaCl in the presence of CHAPS and finally with 4M $MgCl_2$, which is quickly removed by dialysis. This modified version of our published purification scheme is now routinely used on MCF-7 membranes and mouse tissues.

The binding activity recovered from placenta is very low, but on SDS-PAGE analysis using silver stain, we observed a major band of apparent molecular weight of 80,000 to 90,000 daltons as well as some of the 35,000 to 37,000 dalton "core-binding unit" of the Prl receptor purified from mouse liver. The material purified from human placenta is highly cross-reactive on Ouchterlony test plates with rabbit antiserum raised against purified mouse liver prolactin receptor. This antibody reacts only with mouse and human prolactin target tissues, their solubilized membranes and purified receptors. It inhibits the binding of prolactin to solubilized receptors from mouse liver. It does not inhibit the growth promoting effects of Prl on the rat Nb2 cells nor does it substitute for Prl in these cells. This is consistent with a lack of cross-reactivity on Ouchterlony plates between the antibody and rat mammary gland or liver membranes.

The appearance of this 80,000 dalton protein during affinity purification under less denaturing conditions than used previously was confirmed by immunopurification studies. The IgG fraction of rabbit anti-Prl receptor antibody was coupled to a protein-A-Sepharose column and used to purify receptor from mouse livers. By silver staining after SDS-PAGE of this immunopurified receptor, a major band is found at 80,000 to 90,000 daltons. This immunopurified receptor binds lactogenic hormones in a specific manner.

We have now made polyclonal antibodies in rabbits using affinity purified 37,000 dalton "core-binding unit," affinity purified 80,000 to 90,000 dalton receptors and immunoaffinity purifed 80,000 to 90,000 dalton receptors. All antibodies so far have similar properties and show a contiguous precipitation line against solubilized mouse liver on Ouchterlony test plates. The antibodies inhibit lactogen binding to mouse liver microsomes by >80%. They precipitate an 80,000 to 90,000 dalton protein using iodinated immunopurified and affinity purified mouse receptors, iodinated human placental lactogenic hormone receptors purified by the affinity method, and iodinated CHAPS solubilized crude placental membranes. In some cases a minor 37,000 dalton protein is also observed. The antibody can also be used in Western blot experiments to identify an 80,000 to 90,000 protein from solubilized mouse liver membranes and affinity purified receptors.

The polyclonal antibody on hand has been used to identify newly synthesized receptors in mammary glands from in pregnant and lactating mice. Explants of glands were incubated overnight in the presence of ^{35}S-methionine to label the newly synthesized receptors. Extracts of the tissue were then

precipitated with the antiserum against receptor, electrophoresed and fluorographed. The major band of label was found to have a molecular weight of about 80,000 to 90,000 daltons. Some 37,000-40,000 dalton protein was also observed if Prl was present in the labeling medium. We then examined the unlabeled membranes from lactating mammary glands and found that direct immuno-precipitation of CHAPS solubiized receptors primarily produced the higher molecular weight form. However, if the membranes are pre-bound with Prl overnight before solubilization and immuno-precipitation, the 37,000-40,000 dalton core binding unit predominates. These data suggest that the receptor may exist in the membrane as a higher molecular weight form and that during the process of binding to the hormone, it is dissociated to a smaller form including the "core binding unit."

Since the anti-receptor antibody prepared against mouse protein also recognizes the human target tissue receptor, we have developed an immunocytochemical method to detect receptor positive human T47D mammary tumor cells. In preliminary studies, under conditions of growth stimulation of the T47D cell by estradiol and thyroid hormones as many as 10% of the cells appear positive for surface Prl receptors. Without thyroid hormones present in the growth medium no positive cells could be observed. Since the rabbit anti-mouse Prl receptor antibody only weakly interacts with the receptor from human tissues, we decided to examine some of the monoclonal antibodies (MAb) currently on hand in LTIB for their effects on lactogen binding to MCF-7 cells and membranes from solid MCF-7 tumors. We examined IgG fractions from MAb B72.3, B6.2 and B1.1 for their effect on binding of lactogen. Only B6.2 had any effect. In preliminary studies this MAb inhibited binding by over 50%. Since B6.2 is reported to precipitate an undefined 90,000 dalton protein, it is a strong candidate for an anti Prl-receptor antibody.

The recent reports from Sutherland's laboratory that the antiestrogen Tamoxifen (TAM) binds to a membrane associated antiestrogen binding site (AEBS) which is distinct from the classical estrogen receptor, prompted us to examine whether or not this AEBS is related to the prolactin receptor or its action. The MCF-7 cells we grow in our laboratory (non-cloned) respond to human Prl (20 to 200 ug/ml) with a 3-fold increase in cell number after 4 to 5 days in culture. This effect is only seen when the 10% FCS present in the medium has been sufficiently stripped with dextran-coated charcoal to remove over 95% of the endogenous bovine Prl. While the cells do not respond to bPrl under similar culture conditions, this hormone binds to the lactogen receptor and prevents the biologically active human Prl from binding and thus stimulating growth. Under these growth-stimulating conditions, addition of 10^{-6}M TAM to the medium completely inhibits the Prl response. However, since the cells are known to be TAM responsive through the cytosol E_2 receptor, we cannot rule out an E_2-mediated effect.

To examine TAM effects in the absence of E_2 effects, we turned to the Prl-responsive rat Nb2 lymphoma cell line. This cell line does not respond to E_2 in terms of growth. We developed serum-free culture conditions for this cell line so that its growth is completely dependent upon the addition of Prl to the medium. The cells respond to oPrl at concentrations of 5 to 500 pg/ml. This growth is inhibited by >80% by the addition of Prl to the medium. The maximum effective concentration of TAM is 10^{-10}M with no effect on the baseline level of cells maintained

in the absence of Prl. We have demonstrated the presence of membrane-bound TAM AEBS on MCF-7 cells with a Ka of 10^9 M^{-1}. Similar sites on Nb2 cell membranes have a Ka of 10^{11} M^{-1}. TAM AEBS are also present on membranes from lactating mouse mammary glands.

We then examined the direct effect of TAM on binding of lactogenic hormones to their membrane bound receptors. In MCF-7 and Nb2 cells, TAM (10^{-9} to 10^{-6}M) inhibited lactogen binding by 40 to 60%. Using microsomal membranes prepared from MCF-cells, MCF-7 tumors (growing and regressing) and lactating mouse mammary glands, TAM inhibited lactogen binding by >90% in all cases when added directly to the binding reaction. The TAM effect on lactogen binding persists even if the membranes are pretreated with the antiestrogen and then the unbound TAM is removed prior to testing for Prl binding. Prl receptors are found in both golgi and plasma membranes. The greatest effect of direct addition of TAM to the binding reaction is found in purified plasma membranes. The Prl receptors in the plasma membrane are believed to be the biologically active form.

A reciprocal relationship between Prl and TAM binding to membranes was demonstrated by the addition of oPrl to the TAM binding reaction. In the presence of 200 ng Prl, binding of ^{3}H-TAM to AEBS is increased 2X in the membranes from Nb2 and MCF-7 cells. A 3X increase in ^{3}H-TAM binding was demonstrated by adding oPrl (100 ng to 1000 ng) to membranes from mouse mammary glands. Similar results on Prl binding to Nb2 cell and lactating mammary gland membranes and on growth of Nb2 cells were obtained with the antiestrogen Nafoxidine (10^{-10} to 10^{-6}M). From these data we concluded that in addition to its antiestrogen activity, TAM (and Nafoxidine) are antilactogenic. This activity is thought to occur through regulation of the ability of Prl to bind to its receptors on the cell's membrane.

Preliminary data has been obtained which shows that TAM inhibition of Prl binding occurs in solubilized membranes. We also were able to demonstrate a low level of 3H-TAM binding to fractions from an oPrl affinity column used to purify lactogen receptors from solid MCF-7 tumors grown in nude mice. These data suggest that the membrane-bound AEBS may co-purify with the Prl receptor.

Significance to Biomedical Research and the Program of the Institute:

Prolactin-thyroid interactions are important in growth and differentiation of mammary glands. Altered thyroid status may be implicated in changes in risk of human breast cancer. Our studies are aimed at understanding whether changes in thyroid status play a direct or indirect role in mammary tumorigenesis. Thyroid hormones are known to regulate prolactin action through control of lactogenic receptors. Therefore, all aspects of prolactin binding and control of exposed as well as cryptic sites must be examined. Preparation of antibodies against the lactogenic receptors will readily allow us to study the receptor molecule itself in the absence of the hormone and thus determine if lack of prolactin binding in certain mammary tumors is due to lack of receptors or only their existence in masked forms. The use of Prl receptors as a marker for Prl responsive tumors may be of clinical usefulness. Tamoxifen is a commonly used chemotherapeutic agent in breast cancer. Its mode of action in relation to Prl must be defined.

Proposed Course of Research:

We will prepare sufficient quantities of prolactin receptors from human tissue (placenta or solid MCF-7 tumors) using the techniques developed so far. We will prepare polyclonal and, if necessary, monoclonal antibodies against the human receptor.

We will continue to examine the relationship of MAb B6.2 to the Prl receptor by examining its ability to precipitate iodinated CHAPS solubilized membranes from MCF-7 tumors and human placenta as well as affinity purified receptors from these tissues. We will use B6.2 in Western blot analysis of receptors from these sources. If possible, B6.2 will be used to immunopurify the 80,000 to 90,000 dalton protein and we will examine the purified protein for its possible lactogen binding characteristics.

As part of any antibody characterization process for both polyclonal and monoclonal antibodies, we will utilize crosslinking agents. After binding oPrl to receptors (membrane bound, solubilized or purified), we will crosslink the hormone to the receptor using either DSS (disuccinimidyl suberate) or the photoactivated heterobifunctional reagent N-hydroxysuccinimidyl-4-oxidobenzoate. Following SDS-PAGE and Western blotting the receptor-hormone complex will be identified with antibodies against oPrl as well as the putative antireceptor antibodies in parallel. In this way we will unequivocally identify the antibody as an antireceptor antibody.

We will use these antibodies to begin screening human tumors for organ specific receptor content. We will examine primary vs metastatic breast tumors for the presence of receptors by immunocytochemistry. Using the antibody we will examine the regulation of receptor synthesis and availability of this molecule to the hormone in cultured human breast cancer cells (MCF-7 and T47D). Special emphasis will be placed on the role of thyroid hormones in regulating Prl receptors. The relationship of the 37,000 to 40,000 dalton core-binding unit to the larger 80,000 to 90,000 dalton species will be examined using 2D gels, fingerprint analysis of tryptic digests of purified receptors extracted from appropriate regions of one-dimensional SDS-acrylamide gels. We will examine the receptor molecule itself to determine if it is a kinase which is autophosphorylated during hormone binding, as many peptide hormone receptors are now being shown to be. We will continue work on isolation and sequencing the prolactin receptor gene from human and mouse tissues. Using the Prl responsive Nb2 cell and conditions under which MCF-7 cells are Prl responsive for growth, we will examine the effects of Con A and peanut agglutanin (PNA) on lactogen binding and hormonally controlled growth. PNA binding has been proposed as a marker for mammary epithelial cells. In addition, we will purify the membrane-bound TAM receptor using a TAM-affinity column. We will follow both TAM binding and Prl binding to the protein fractions throughout the procedure to determine if they co-purify. Clearly establishing the relationship of TAM-AEBS on membranes to Prl-receptors and Prl action will be of high priority because of its implications in chemotherapy.

Publications:

Vonderhaar, B.K., Bhattacharya, A., Alhadi, T., Liscia, D.S., Andrew, E.M., Young, J.K., Ginsburg, E., Bhattacharjee, M. and Horn, T.M.: Isolation, characterization and regulation of the prolactin receptor. J. Dairy Science 68: 466-488, 1985.

Bell, J.A., Moffat, K., Vonderhaar, B.K., and Golde, D.W.: Crystallization and preliminary x-ray characterization of bovine growth hormone: purification of bovine prolactin and growth hormone. J. Biol. Chem. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08226-09 LTIB formerly Z01 CB 08226-08 LPP

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Hormones and Growth Factors in Development of Mammary Glands & Tumorigenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

B.K. Vonderhaar	Research Chemist	LTIB, DCBD, NCI
W. Kidwell	Chief, Cell Cycle Regulation Section	LTIB, DCBD, NCI
T. Horn	Staff Fellow	LTIB, DCBD, NCI
R. Callahan	Chief, Oncogenetics Section	LTIB, DCBD, NCI
E. Ginsburg	Biologist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. H. Nakhasi, Bureau of Biologics, FDA
Dr. Sandra Haslam, Michigan State University, East Lansing, MI

LAB/BRANCH: Laboratory of Tumor Immunology and Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.25	0.75

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project is designed to understand the role of hormones and growth factors in normal mammary gland development and differentiation. Studies include: 1) examination of the role of epidermal growth factor and mammary gland-derived growth factors in lobulo-alveolar development of the mouse mammary gland, 2) defining the roles of estrogen and progesterone in priming the mammary tissue prior to whole organ culture to determine their effects on induction of EGF receptors, mammary gland-derived growth factor receptors and the production of growth factors by the animals, 3) examine the hormonal conditions in vitro which induce production of autocrine growth factors by normal mammary tissue and breast cancer cell lines.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

The purpose of these studies is to investigate the role of various hormones, vitamins, and growth factors in mammary gland development, differentiation and tumorigenesis. The roles of prolactin, thyroid hormones, adrenal steroids and epidermal growth factor (EGF) and mammary derived growth factor (MDGF) in lobulo-alveolar development of the mouse mammary glands in whole gland culture and in vivo was examined. The purpose was to define the conditions involved in normal and hyperplastic development of the gland and production of mammary-derived growth factors.

Methods Employed:

C3H/HeN and BalbC mice were used. Whole gland and organ culture was performed using chemically defined serum-free medium supplemented with various hormones, growth factors, and metabolic inhibitors. Lobulo-alveolar development of mammary glands was assessed by hematoxalin staining of whole mounted glands. Estrogen/progesterone (E/P) priming of 4 week old BalbC mice was performed by inserting a pellet (cholesterol: progesterone: estradiol 1001:1000:1) or E alone or P alone at the same relative concentrations under the skin of the animals for the indicated period of time. Mammary-derived growth factor (MDGF) was isolated from mammary tissue by homogenization in PBS containing 2% Triton X-100 followed by centrifugation at 20,000 xg for 20 min or by acid-alcohol extraction as reported by Kidwell. Purified human milk derived growth factor (hMF) was from Dr. Kidwell. EGF levels and MDGF concentration in the tissue extracts were determined by standard RIA techniques and competition for ^{125}I-EGF binding to hepatic microsomal membranes. Analysis of EGF and hMF binding to glands of mice was performed by the method of Scatchard. Altered thyroid states were induced by placing either 0.1% thiouracil (hypothyroid) or 2 µg/ml T_4 (hyperthyroid) in the drinking water.

Major Findings:

Whole gland cultures of immature mouse mammary glands was used to assess the impact of epidermal growth factor (EGF) and mammary derived growth factors (MDGF) on lobulo-alveolar development of the glands in the presence of insulin (I), prolactin (Prl), aldosterone (A) and hydrocortisone (H).

4 week old BalbC mice were implanted with E/P or E or P pellets. At various times the #2 thoracic glands were removed and cultured in the presence of IHPrlA ± EGF or MDGF. After as long as 9 days of priming with the pellet, no detectable differences in mammary gland morphology were detected between primed and unprimed glands. Glands from animals primed for 9 days or longer had extensive lobulo-alveolar development after culturing with IHPrlA. However, when animals were primed for 6 days, the glands could develop only if 60 ng/ml of EGF was added to the culture media. This requirement for EGF was equally met by addition of a MDGF. Platelet derived growth factor and fibroblast growth factor do not substitute for EGF. MSA will partially substitute for EGF. Neither E or P alone during priming (even for 9 days) allowed the glands

to develop in the presence of the hormones in vitro.

We examined the possibility that the mammary gland in primed animals contains a growth factor for which EGF substitutes in culture. These studies were prompted in part by observations using anti-EGF antibodies during priming and culture. If mice are injected with anti-EGF during the 9 days of priming with E/P, no effect on subsequent lobulo-alveolar development was seen in the presence of IHPrlA. In addition, after E/P priming for 9 days, full lobulo-alveolar development occurs in response to IHPrlA even in the presence of anti-EGF. Attempts to "prime" the animals with EGF in vivo were unsuccessful. These data suggested that the active growth factor is not EGF, but an EGF-like growth factor. Therefore induction of EGF and MDGF receptors was examined under priming conditions. Glands from E/P primed animals were tested for their ability to bind ^{125}I-EGF. Glands from 6 day primed animals bound 15-20 times more EGF/mg tissue than glands of unprimed mice. EGF binding was specific to epithelial rich regions of the gland and undetectable in fat pad free of epithelium.

Scatchard analysis of the binding of EGF to the epithelial rich region of E/P primed glands showed 2 classes of receptors with Kas of $1.25 \times 10^{9} M^{-1}$ and $3.6 \times 10^{9} M^{-1}$. These values are very similar to those reported by others for epithelial cells of mid-pregnancy and lactating mammary glands. Both E and P alone were able to induce the EGF receptors on this tissue.

Using purified human milk derived growth factor from Dr. W. R. Kidwell, we examined the ability of E/P or E or P alone during priming to induce the growth factor receptors. Only E priming resulted in an increase in hMF receptors. P had no effect. By Scatchard analysis of binding data, the receptors were found to have a Kd of 2.5×10^{-9}M for the hMF. There are 10 pmoles of factor bound per mg membrane protein. The EGF receptor induced by E priming recognized the hMF at about 30% the level of EGF while the hMF receptor bound EGF at less than 10% the level of hMF.

MDGF from E/P primed tissue was added to cultures of 6 day primed glands in the presence of IHPrlA. Lobulo-alveolar development was seen in the glands at a concentration of 0.3 ng/ml of MDGF (based on EGF binding competition). Purified hMF was also able to stimulate glandular development but 50 ng/ml was required.

Thus, we tentatively concluded that E/P priming enhances the synthesis of a MDGF and its receptors on mammary epithelial cells of immature female mice. Receptor induction is the result of the action E. We do not yet know the role of P nor do we know which hormone(s) are necessary for induction of the MDGF. Neither E nor P alone can prime the gland sufficiently (even after 9-11 days) to respond to IHPrlA + EGF in terms of lobulo-alveolar development. Addition of E and P to the culture medium along with IHPrlA and EGF (or MDGF) cannot promote lobulo-alveolar development of unprimed glands. It is possible that yet another factor (hormone) is involved which is supplied by the host animal during priming. This hormone may be involved in inducing the receptor for the growth factor.

Induction of EGF receptors in various target tissues has been shown to be regulated by thyroid hormones. Since lobulo-alveolar development of the mammary gland both in vivo and in vitro as well as functional differentiation is subject to variation in different thyroid states, we looked at the regulation of EGF receptors in mammary glands of mice at various stages of development by alteration in thyroid status in vivo. At all stages examined, (i.e., 5-10 week old growing glands, young mature virgin, i.e., 3 month old, and adult virgin and primiparous involuted glands, 10 month old), the binding of EGF to the mammary glands from mildly hypothyroid mice was reduced by about 75% compared to euthyroid controls. This inhibition could be reversed by brief treatment of the hypothyroid animals with T_4. The inhibition is due to a decrease in the higher affinity binding site.

Our conclusions concerning the role of E/P in MDGF action are supported by preliminary results using cholesterol:EGF or cholesterol:MDGF pellets inserted directly into the glands of 5 week old mice and allowed to remain in vivo for 3 to 5 more days. Neither type of pellet alone resulted in glandular development in vivo. Only when an E/P pellet was simultaneously placed subcutaneously in the interscapular regions did lobulo-alveolar development of the EGF or MDGF containing glands occur. The subcutaneous E/P pellet alone had no effect on the glands' development nor did pellets made from extracts of unprimed glands inserted directly into the gland.

Significance to Biomedical Research and the Program of the Institute:

Prolactin-thyroid interactions are important in growth and differentiation of mammary glands. Altered thyroid status may be implicated in risk of human breast cancer. It is not yet clear from the literature whether the effects of thyroid hormones are primary (i.e., directly on the mammary gland) or secondary (i.e., through alterations in other hormones). Therefore, all aspects of thyroid hormone control of growth and differentiation of the normal gland as well as development of mammary tumors must be understood. The development of hyperplastic alveolar nodules (HAN) in mouse mammary glands has been correlated with subsequent mammary tumor development. Thus, we wish to examine those growth factors (both from normal and tumor tissue) which lead to lobulo-alveolar development and HAN formation in mammary glands in culture. The ability of unprimed tissue to respond to hormones and growth factors in defined culture medium affords us an unique opportunity to study mechanisms for controlling development and differentiation of the HAN.

Proposed Course of Research:

We will continue to study the nature of the MDGF and the induction of the receptors for EGF and this factor. Mammary tissue from different growth stages and tumors will be extracted and tested for the ability to support lobulo-alveolar development in vitro as well as in vivo with pellets inserted directly into the gland. We will continue to define the individual roles of E and P in the priming process by examining growth factor and receptor induction by each alone. We will also examine the ability of each hormone alone to support the

local growth factor induced development of the gland in vivo. These studies should ultimately aid us in promoting lobulo-alveolar development in vitro using unprimed glands. To this end, we will continue our studies on induction of EGF and EGF-like growth factor receptors in normal mammary glands by thyroid hormones. This will now be attempted in vitro using the organ culture method previously used to study regulation of the Prl receptor.

In collaboration with Dr. David Salomon, we propose to examine the production of α-TGF's by E/P primed glands and by the normal glands of mice from various growth and differentiation stages. This factor will be looked for in tissue extracts and in conditioned medium from glands cultured with various hormone combinations. A possible role of α-TGF's in the priming process will be examined as well as during subsequent culture periods by utilizing commercially available anti-α-TGF antibodies. As we did with anti-EGF, we will give animals anti-α-TGF during priming with E/P or during culture and determine the subsequent lobulo-alveolar development of the gland.

In the long run, we plan to utilize the information on hormonally controlled development of the gland and of preneoplastic lesions, in a similar system using human tissue. Hopefully, we will develop conditions for in vitro development of unprimed tissue. If not, it may be possible to "prime" human tissue from reduction mammoplasty and/or benign lesions or normal areas of biopsy samples - by passage through nude mice supplemented with priming hormones. Such a heterologous priming system will be developed using bovine tissue.

This whole organ culture system can also be used to study induction of hyperplastic alveolar nodules (HAN) in vitro by carcinogens (first shown by Mihir Banerjee). This nodule inducing system, first with mice and eventually with human tissue, will be used under various hormonal conditions in vitro to examine growth factor induction and oncogene activation. These studies will be performed in collaboration with Dr. Robert Callahan's group.

Publications:

Vonderhaar, B.K.: Hormones and growth factors in mammary gland development. In Veneziale, C.M. (Ed): Control of Cell Growth and Proliferation. Van Nostrand, Reinhold and Co., Inc., New York, 1984, pp. 11-33.

Bhattacharjee, M. and Vonderhaar, B.K.: Thyroid hormone enhance the synthesis and secretion of alpha-lactalbumin by mouse mammary tissue in vitro. Endocrinology 115: 1070-1077, 1984.

Vonderhaar, B.K. and Bhattacharjee, M.: The mammary gland: a model for hormonal control of differentiation and preneoplasia. In Mihich, E. (Ed.): Biological Responses in Cancer: Progress Toward Potential Applications, Vol. III. Plenum Publishing Co., New York, 1985 (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08288-03 LTIB
formerly
Z01 CB 08288-02 LPP

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
cDNA Cloning of N-acetylglucosaminide Beta-1→4 Galactosyltransferase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. Qasba	Research Chemist	LTIB, DCBD, NCI
S. Matarazzo	Staff Fellow	LTIB, DCBD, NCI
H. Naramatsu	Guest Researcher	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*
Drs. H. Okayama and I. David, LMG, NICHD
Drs. K. Brew and S. Sinha, Dept. of Biochem., Miami Med. School, Miami, Fla.

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Galactosyltransferases are the family of enzymes which transfer galactose from UDP-gal to the non-reducing residues of oligosaccharides of various glycoconjugates as well as monosaccharides. The most common sequence, Gal-beta-1→4GlcNAc, which occurs in glycolipids and as well as in the O-and N-linked glycoprotein oligosaccharides, is formed by N-acetylglucosaminide beta-1→4 galactosyltransferase. This enzyme has been purified from bovine and human milk, where it interacts with alpha-lactalbumin to form lactose synthase. Alpha-lactalbumin modifies the activity of this galactosyltransferase in such a way that inhibits the transfer of galactose from UDP-galactose to N-acetylglucosamine, either free or linked as a terminal sugar of glycoprotein but facilitates the transfer to glucose or myoinositol.

To understand the modulation of the galactosyltranferase activity essential for generating specific cell-surface antigenic determinants, we have undertaken the isolation and characterization of galactosyltranferase cDNA clone. Commercially available bovine galactosyltransferase was repurified, and after acetylation of lysine residues subjected to tryptic digestion. Some of the tryptic peptides were sequenced. A mixed 21-mer and 27-mer nucleotide probes corresponding to two different peptides were synthesized and used as hybridization probes. A bovine cDNA library was constructed in a Okayama-Berg expression vector from the lactating bovine mammary gland poly $(A)^+$RNA. This cDNA library was screened with the labeled oligomer probes. Clones hybridizing to both the probes were further analysed. Authenticity of gal. transf. clone was confirmed by a) translation of the hybrid selected mRNA in frog oocytes and measuring gal. transf. activity and b) transfecting Cos cells with the cDNA clone and measuring transferase activity.

Project Description

Objectives:

The purpose of this work is to a) to isolate and characterize the cDNA clone for N-acetylglucosaminide β1→4 galactosyltransferase, b) to know the general structural features of the family of enzymes of galactosyltransferases, c) to study the expression of the gene during development and differentiation and d) the organization of the gene during early fetal development, in normal adult, in metastasising and non metastasising tumors.

Methods Employed:

1) A cDNA library was constructed in a Okayama-Berg expression vector from the lactating bovine mammary gland poly $(A)^+$RNA. This vector permits the expression of the cDNA in mammalian cells.

2) A commercially available galactosyltransferase (sigma) was repurified by affinity chromatography on bovine α-LA sepharose column. Purified protein was acetylated to block the lysine groups and then digested with trypsine. Some tryptic peptides were separated on HPLC and sequenced. 21mer and 27mer mixed synthetic oligonucleotide probes corresponding to two tryptic peptides were synthesized.

3) Bovine cDNA library made in Okayama-Berg vector was screened with the synthetic nucleotide probes. Clones which hybridized to both the probes were further analyzed by: a) translating in frog oocytes the hybrid selected mRNA and subsequently measuring the gal. transferase enzymatic activity; b) transfecting the Cos cells with the plasmid DNA and measuring the expression of gal. transferase by enzymatic activity.

Major Findings:

Galactosyltransferase (GT) purified either from cow or human milk is a single polypeptide chain of about 55K daltons. The amino terminal end of GT is blocked. Highly purified GT-preparations, which have been twice purified on α-lactalbumin affinity columns have been shown at times still to carry some tightly bound contaminants such as IgG and lysozyme. Thus, some of the partial peptide sequences that one might get even from a highly purified preparation may then sometimes represent sequence(s) of the contaminating protein. Since the nucleotide probes are made against randomly chosen peptide sequences and used for selecting the cDNA clones, it is then essential to prove the authenticity of the cDNA clone by the method(s) which would either measure the enzymatic activity of the encoded protein or by the alignment of cDNA sequence with most of the peptide sequences. For these reasons we chose the following methods: 1) We constructed the cDNA library in Okayama-Berg expression vector. Expression of the cDNA sequences in this vector in mammalian cells is under the control of SV40 promoter and enhancer. Transfection of Cos cells with a full length cDNA clone should encode a protein whose enzymatic activity can be measured. 2) Foreign mRNAs

microinjected into frog oocytes have been shown to be very efficiently translated. mRNA hybrid selected to a GT cDNA clone and injected into frog oocytes should code for a protein with a measurable enzymatic activity.

Construction and screening of bovine cDNA library. A cDNA library of $3x10^6$ independent colonies was generated from the lactating bovine mammary gland total poly $(A)^+$RNA by the vector-linker method of Okayama and Berg. This cDNA library was screened twice, approximately 60,000 clones each time, with two oligonucleotide mixed probes, 21mer and 27mer. From 60,000 clones, fifteen clones hybridized individually with these probes, only three out of these hybridized with both the probes. All the three showed similar restriction maps and the longest clone out of these has a cDNA insert of 3.8kb. Expression of GT in Cos cells after transfection with these clones and mRNA hybrid selected with these clones and microinjected in frog oocytes for measuring the enzymatic activities is currently being studied.

Significance to Biomedical Research and the Program of the Institute:

Galactosyltransferases play a significant role in the synthesis of cell surface antigenic determinants in normal and neoplastic cells. The carbhoydrate sequence Gal. $\varepsilon\beta1\rightarrow4$ GlcNAc, synthesized by N-acetylglucosaminide $\varepsilon\beta1\rightarrow4$ galactosyltransferase, occurs in glycoproteins and glycolipids, and is recognized as developmentally regulated antigenic determinants of several cell types. A cDNA clone will be a useful tool for studies on the regulation of expression of this gene in normal and neoplastic cells.

Proposed Course of Research:

A) To isolate human and mouse GT clone using the bovine GT clone as a hybridization probe; B) to complete the sequence analysis of human and mouse GT clones; c) study the regulation of the expression of galactosyltransferase gene during development and differentiation; and d) study the organization of the gene during early fetal development, in normal adult, in metastasising and non metastasising tumors.

Publications:

Qasba, P. K., Hewlett, I. K. and Byers, S.: The presence of the milk protein α-lactalbumin and its mRNA in rat epididymis. Biochem. Biophys. Res. Comm. 117: 306-312, 1983.

Byers, S., Paulson, H. L., Qasba, P. K. and Dym, M.: Cytochemical localization of α-lactalbumin-like molecules in rat epididymis. Biol. Reprod. 30: 171-178, 1984.

Byers, S., Dym, M., Hewlett, I. K. and Qasba, P. K.: α-lactalbumin-like proteins in the male reproduction tract. Ann. N.Y. Academ. Sci. Hormone Action and Testicular Function, NICHD workshop 438: 8-17, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08220-04 LTIB
formerly
Z01 CB 08220-03 LPP

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Sequence of Galactosyltransferase Modifier Protein Alpha-lactalbumin and Wp

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. Qasba	Research Chemist	LTIB, DCBD, NCI
S. Matarazzo	Staff Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

None

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

(A) Alpha-lactalbumin (alpha-LA)-like proteins modify the activity of N-acetyl-glucosaminide beta 1-4 galactosyltransferase and their expression is regulated by hormones. We have isolated and sequenced entire mammary gland alpha-LA gene, including its 5'-flanking sequences. The gene sequences were compared with chicken lysozome gene, since it was proposed that the two genes have arisen from a common ancestral gene. These studies show: 1) alpha-LA and lysozome genes contain 3 introns at similar positions. The first three exons show high nucleotide homologies; 2) 5'-flanking sequence of alpha-LA gene has several identical short repeat sequences. Some of these sequences resemble the concensus sequences which bind hydrocortisone and progesterone receptors which modulate the expression of this gene; and 3) the fourth exon of alpha-LA, which partly codes for the C-terminal residues of the protein, essential for its interaction with galactosyltransferase, is markedly different from the corresponding exon of lysozome gene and is preceded by two (TG) repeats-$(TG)_{21}$ and $(TG)_{24}$. It is suggested that the 4th exon of alpha-LA coding for a new functional unit might have replaced the DNA region of primordial lysozome gene and led to a protein with a new function. (B) Rat whey phosphoprotein (Wp) has a double domain structure with striking homologies to a protease inhibitor. Wp-gene expression is regulated by the same set of hormones as the alpha-LA gene. Rat Wp-gene has been isolated and its full sequence is nearing completion. The gene structure and nucleotide sequences show that: a) there are three intervening sequences in the entire gene which is 2.5 kb in length; b) second and third exons code for first and second domains of the protein, respectively; and c) first and the third intervening sequences encompassing the second and third exons, have several repeat and inverted repeat sequences.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

The purpose of this work is to: a) determine the entire DNA sequence of α-LA and whey phosphoprotein gene; b) compare gene sequences of α-LA with lysozome and of whey phosphoprotein with known protease inhibitors; c) compare 5'-flanking and intervening sequences of α-LA and Wp-genes, since their expression is regulated by the same set of hormones; d) determine the relationship, if any, between the exons and protein domain structures; and e) study modification of gene sequences upon mammary tumorigenesis.

Methods Employed:

1. The amplified rat liver DNA library (in Charon 4A phage DNA) was screened using the in situ plaque hybridization techniques of Benton and Davis (Science 196: 180-187, 1977). Plaques containing structural gene sequences for α-LA and Wp were purified as described by Maniatis et al., (Cell 15: 687-701, 1978).

2. For restriction enzyme mapping DNA is digested and fragments separated on agarose gels. After visualization of the DNA bands by ethidium bromide staining, DNA is analyzed by Southern blots. Nick-translated [^{32}P] labeled α-LA and Wp cDNAs are used as hybridization probes to localize the structural gene sequences for α-LA and Wp. 5'-End labeled mRNAs are used as hybridization probes to localize the 5'-end sequences.

3. DNA sequence analysis is carried out as follows: DNA restriction fragments are sequenced, a) after labeling at 3'- or 5'-ends by the chemical method of Maxam and Gilbert or b) by M13mp11 dideoxy sequencing technique of Messing et al., (Nucleic Acid Res. 9: 309-321, 1981).

Major Findings:

(A) Gene sequence of galactosyltransferase modifer protein, the mammary gland α-lactalbumin.

I. Similarities between α-LA and lysozome.

α-LA gene of 2.5 kb contains three intervening sequences which are at similar positions within the coding regions as in chicken egg white lysozome gene. The intervening sequences in the two genes differ in lengths. All the introns in both the genes not only begin with GT dinucleotide and end with AG, sequences thought to be necessary for correct RNA splicing of various eukaryotic genes, but also have additional common sequences at the exon/intron junctions. Exons 1, 2 and 3 of the two genes (165, 159 and 76 bp for α-LA gene and 165, 162 and 79 bp for lysozome gene) show high nucleotide homologies.

II. Exon coding for the functionally important domain of α-LA is divergent from the corresponding region of the lysozome gene.

Fourth exon of α-LA and lysozome differ in length and show least similarities. Thus, although there is a high degree of similarity implying homology between the other exons, the two genes have diverged in the region of the fourth exon, since this region of the α-LA gene shows no statistically significant similarity with the corresponding lysozyne exon. Conformation of the main chain of α-LA, based on model building, though for the most part similar to lysozyme, differs from it from residue 108 onwards. These residues are encoded by the fourth exon of the two genes which show least similarity. In α-LA, peptide region from residue 105 onwards, contains amino acids essential for its interaction with galactosyltransferase and is conserved among species variants of α-LA. This functionally important region of α-LA is conformationally different from the corresponding region of lysozyme and is coded by a separate nucleotide region preceded by two $(TG)_n$ repeats. The observed differences in the fourth exon of the two genes suggest that this exon of the primordial gene either diverged more rapidly than the other exon through accumulation of point mutations, insertions, or deletions, or according to the split gene hypothesis, the entire fourth exon might have been replaced by a new region of DNA, generating a DNA sequence which partly coded for a new functional unit of a protein. TG dinucleotide repeats, present in the intervening sequence preceding the fourth exon of the αLA gene, have the potential of forming a Z-DNA structure which have been implicated in genetic recombination, rearrangement and regulation of gene expression, and may have played a role in generating the observed differences in the fourth exons of the two genes.

III. 5'-Flanking sequence of α-LA contain several repeat and potential receptor binding sequences.

There is an A-rich sequence, 5'-TAAATAAA-3', at position -25 to -17 of cap site, similar but not identical to the concensus sequence TATAAA, found at the initiation site of many other genes. A number of almost identical short repeat sequences preceding this A-rich sequence appear only in the 5'-flanking region of this gene. Sequence 5'-GGGANNNTGGCAG-3' is repeated two times and the hexanucleotide sequence TGGCAG four times within the position -90 to -190. A binding protein to pentanucleotide sequence TGGCA has been shown to be present in several mammalian cells. The significance of this sequence for the α-LA gene expression is being currently investigated. A decanucleotide sequence CCGTGCCCA is repeated at position -508 and -1236. A potential hydrocortisone and progesterone receptor binding site is repeated two times at position -348 and -714. Also it is worth noting that the nanonucleotide sequence ATCCCTTTC, which appears at position -498 to -489 and at position -390 to -381, resemble the first nine nucleotides of the nineteen nucleotide consensus sequence $ATC^{C}_{T}\,^{C}_{T}ATT^{A}_{T}TCTG^{G}_{T}CTTGTA$, thought to be involved in the progesterone receptor recognition site of the ovalbumin gene.

(B) Gene structure and sequence of whey phosphoprotein (Wp):

I. Correlation of the protein domain structure with the gene structure.

A recombinant λ-phage carrying approximately 18kb of rat DNA contains whey phosphoprotein gene which is about 2.6kb in length. The gene contains three intervening sequences nearly equidistant from each other within the coding region. Two EcoRI fragments 2.5 and 3.3kb in length contain the entire gene. The 2.5kb

fragment contains 5'-flanking sequence and the first exon. Second, third and the fourth exon are present in the 3.3kb EcoRI fragment. The two domains of Wp-protein are separately coded by the 2nd and 3rd exon. The intervening sequence which bound these two exons have several repeat and inverted repeat sequences.

II. Features of 5'-flanking and intervening sequence.

About 750bp of the 5'-flanking region have been sequenced. The sequence contains a potential promoter site, TTTAAAT, as well as several potential binding sites for the glucocortcoid receptors. At -700 position there is a long T stretch. The intervening sequence before the 2nd exon, later coding for the first domain of Wp-protein, has TATAAT sequence, several short repeat and inverted repeat sequences, as well as $G_{(n)}T_{(n)}$ repeat. Third intervening sequence following the third exon, later coding for the 2nd domain of Wp-protein, has also several repeat and inverted repeat sequences.

Significance to Biomedical Research and the Program of the Institute:

The information about the gene structure and sequence of the mammary gland α-LA shall help in understanding the modifier activity of this protein towards galactosyltransferase and also the hormonal regulation of the expression of this gene in normal and neoplastic transformation of the mammary cells. Comparison of these sequences with the whey phosphoprotein gene sequence which is also regulated by similar hormones shall help in defining the sequences which interact with hormonal receptors and regulatory proteins for gene expression.

Proposed Course of Research:

To complete the remaining sequences of the Wp protein gene.

Publications:

Qasba, P. K. and Safaya, S. K.: Structure and nucleotide sequence of the rat α-LA gene: Comparison with the chicken egg white lysozyme gene. Nature 308: 377-380, 1984.

Chomczynski, P. and Qasba, P. K.: Alkaline transfer of DNA to plastic membrane. Biochem. Biophys. Res. Comm. 122: 340-344, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08286-03 LTIB
formerly
Z01 CB 08286-02 LPP

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Sequences Regulating the Expression of Gal. Transf. Modifier Protein

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. Qasba	Research Chemist	LTIB, DCBD, NCI
S. Matarazzo	Staff Fellow	LTIB, DCBD, NCI
H. Lubon	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

None

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	1.75	0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Alpha-lactalbumin (alpha-LA)-like proteins modify the activity of N-acetylglucosaminide beta 1-4 galactosyltransferase. Mammary gland alpha-LA bears sequence homology with lysozyme. In mammary gland alpha-LA gene expression is regulated by insulin, hydrocortisone, prolactin and progesterone. Previously we have sequenced mammary alpha-lactalbumin gene and shown that: a) 5'-flanking sequence has several short repeat sequences and some of which are similar to the concensus sequence for the binding site of hydrocortisone and progesterone receptor; b) the functional domain of alpha-LA which contains amino acids essential for its interaction with galactosyltransferase, and is conformationally different from the corresponding region of lysozyme, is coded by a separate nucleotide region preceded by two $(TG)_n$ repeats. In the present studies the regulatory elements of alpha-LA gene are being localized. Various regions of alpha-LA gene have been introduced into chloramphenicol (CAT)-vectors and tested in mammalian transfection experiments for their ability to influence CAT-expression. Alpha-LA gene sequences which become DNAase hypersensitive and bind specific proteins during different stages of mammary gland development have been studied. The results show: a) 700 bp DNA fragments from the 3'-end of alpha-LA gene, which contains most of the third intervening sequence with its two $(TG)_n$ repeats - $(TG)_{21}$ and $(TG)_{24}$ - and a part of the 4th exon which codes for the functionally important region of alpha-LA, acts in an enhancer fashion in CAT-assays; (b) activity of this enhancer is cell type specific; (c) 5'-flanking sequences of alpha-LA gene show DNAase hypersensitive regions during different stages of mammary gland differentiation; d) nuclear protein fractions extracted from mammary gland nuclei bind to specific regions of 5'-flanking sequence of alpha-LA gene.

Project Description

Objectives:

To identify: a) the promoter and enhancer sequences of α-LA gene; b) the protein factor(s) which bind to α-LA gene sequences and modulate its expression; and c) the chromatin structure of α-LA gene during functional differentiation of mammary cells.

Methods Employed:

1. Construction of the plasmids carrying rat α-LA gene sequences: DNA fragments of various regions of α-LA gene were inserted into the Bgl II site of pA_{10} Cat_2 and pCat T_{3m} vectors which contain bacterial chloramphenicol acetyltransferase (Cat) gene.

2. Mammalian cell cultures and DNA transfection: The following cells have been transfected with Cat constructs:

a) Primary epithelial cell cultures from mid-pregnant rat mammary gland cells grown with and without collagen substratum.

b) continous cell lines having insulin and hydrocortisone receptors: e.g. rat hepatoma cell line, H4IIE cells; mouse mammary cell line, comma D cells; mouse mammary tumor cell line, MMT-cells; human breast tumor cell lines, T-47D and MCF-7. Plasmids were transfected onto cells 70% confluent by a modification of the Ca^{2+}-precipitation method described by Gorman et al. CAT activity was measured from the cell extracts by standard procedures.

3. Nuclear extracts and DNA binding: Nuclei were isolated by a standard method and extracted with high salt. End labeled restriction fragments are incubated with the nuclear extracts and the binding measured by nitrocellulose filter assay. Nuclease hypersensitive regions of α-LA gene are measured by the indirect labeling method of C. Wu.

Major Findings:

I. Regulatory elements of rat α-LA gene.

We used a convenient vector system to test the regulatory elements of α-LA gene. This involves linking a putative regulatory control region to an enzymatic "reporter" function. pCat T_{3m} contains the bacterial gene coding for the enzyme chloramphenicol acetyltransferase (CAT) and an SV40 polyadenylation signal, but not the SV40 TATA box, 21-bp repeats, or an enhancer element. Since CAT activity is normally absent from mammalian cells, the promoter or promoter cum enhancer activity of fragments cloned into the vector are quantified by measuring CAT activity in transfected cells. pA CAT_2 vector contains the SV40 early promoter directing transcription of the CAT gene but does not contain a functional SV40 72-bp enhancer sequence. This vector is designed then to measure the enhancer activity of the inserted sequences by measuring CAT activity in transfected cells. A positive control, pSV2-CAT, contains the SV40 enhancer and promoter sequence.

Construction and transfection of the chimeric plasmids.

Most of the rat α-LA gene sequence is present on 2.57 and 0.95kb EcoRI fragments. The 2.57kb EcoRI fragment contains the 5'-end of the gene and has a BamHI site in the middle where also the transcriptional start site is located. Thus from the 2.57kb EcoRI fragment BamHI generates two fragments: 1) 1.25kb EcoRI-BamHI 5'-flanking sequence, and 2) 1.32kb BamHI-EcoRI fragment. The 1.25kb fragment contains putitative promoter sequence, TAAATAAAA, several short repeat sequences and potential hydrocortisone and progesterone receptor binding sites. The 1.32kb BamHI-EcoRI fragment contains 1st, 2nd and 3rd exon, and the 1st and 2nd intervening sequences, and 150 bp of 3rd intervening sequence. The 0.95kb EcoRI fragment of the α-LA gene contains most of the 3rd intervening sequence and a part of the 4th exon. It is cut with BamHI, which generates 0.22kb 5'-end and 0.73kb 3'-end fragment. The 0.73kb BamHI-EcoRI fragment contains two $(TG)_n$ repeats and a part of the 4th exon. Thus α-LA gene was conveniently divided into three DNA segments - 1.23kb EcoRI-BamHI 5'-flanking sequence, 1.32kb BamHI-EcoRI fragment and 0.73kb BamHI-EcoRI fragment. These DNA fragments were inserted into the Bgl II site of pCat T_3m and $pA_{10}Cat_2$.

Enhancer sequence of α-LA is at the 3'-end of the gene.

Out of all the fragments tested, only 0.73 BamHI-EcoRI fragment inserted into pCat T_3m vector in 3'-5' orientation, enhances the CAT gene activity upon transfection of several different cell types. pCat T_3m and $pA_{10}Cat_2$ vectors by themselves showed no detectable activities. The enhancement of CAT gene expression, however, was dependent on the type of cells transfected. Activities were in the following order: MMT-cells > T-47D > Comma-D > H4IIE. CAT activity was not detectable in H4IIE transfected cells. pSV_2Cat which contains SV40 promoter and enhancers on the other hand, induced significant CAT activity in all cell types. In MMT-cells 0.73kb BamHI-EcoRI fragment inserted into pCat T_3m vector in 5'-3' orientation showed highest CAT activity. The BamHI-EcoRI fragment 0.73kb, contains two $(TG)_n$ repeats of the third intervening sequence and the part of the fourth exon which codes for the functionally important C-terminal portion of α-LA. Fourth exon of α-LA which has either diverged or been replaced from the primordial gene of α-LA and lysozyme, is thus preceded by a seequence which acts in an enhancer fashion.

DNAase hypersensitive regions and nuclear binding protein(s).

Nuclear extracts prepared from the rat mammary gland bind certain end labeled restriction fragments generated from 1.25kb EcoRI-BamHI 5'-flanking fragment of α-LA gene. This binding activity is maximum in the extracts from the mammary gland of mid-pregnant rat. DNAase hypersensitive regions were also detected in the 5'-flanking sequence. Proportion of this DNAase hypersensitive fraction of α-LA gene in the nuclei isolated from the mammary gland increases from midgestation to lactation.

Significance to Biomedical Research and the Program of the Institute:

Since galactosyltransferase plays a very significant role in the synthesis of cell surface antigenic determinants in normal and neoplastic cells, and α-Lactalbumin modulates its activity, it is essential to know more about the functional domain of this modifier protein and the regulatory elements of this gene which control the expression of this gene.

Proposed Course of Research:

1) To narrow down the sequence acting as promoter and enhancer. 2) To identify the DNAase hypersensitive regions of the entire α-LA gene. 3) To identify the DNA sequences to which specific protein factor(s) bind. 4) To identify the proteins involved in the transcriptional activity of the gene.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08293-02 LTIB
formerly
Z01 CB 08293-01 LPP

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Essentiality of Insulin for the Accumulation of Rat Milk Protein mRNA's

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. Qasba Research Chemist LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*
Y. Topper, Chief, LBM, NIADDK
P. Chomczynski, Visiting Scientist, LBM, NIADDK

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

The control of overt differentiation of mammary gland in vitro involves an interplay between polypeptide and steroid hormones. Insulin, hydrocortisone and prolactin are absolutely required for the expression of milk protein genes. Role of individual hormones is being studied. These studies have shown: 1) In the presence of glucocorticoid and prolactin insulin is absolutely required for the expression of milk protein genes. In the present study it was shown that the accumulation effect on the 25K rat casein mRNA does not reflect stabilization of the transcript by insulin. Rather, insulin is essential for its synthesis in the presence of glucocorticoid and prolactin. Thus, insulin can be considered as a developmental hormone in the mammary system. Neither fetal calf serum nor Multiplication stimulating activity (MSA) or epidermal growth factor (EGF) can substitute insulin effect on differentiation, though these hormones can sustain mammary cell vability in culture. 2) There is absolute requirement of hydrocortisone for accumulation of these messages, specifically the accumulation of 42K casein mRNA in mammary tissue from adrenalectomized, virgin rat is almost 20x higher in the presence of exogenous hydrocortisone than in its absence. Accumulation of 25K casein mRNA is also totally dependent on the steroid.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

The purpose of this work is to define the role of insulin and prolactin on the synthesis and accumulation of casein and α-lactalbumin mRNA in rat mammary gland.

Methods Employed:

1. Mammary explant cultures originally described by Elias (Science 126: 842, 1957) and further improved by Dr. Y. Topper's group.

2. Rat cDNA clones isolated, characterized and sequenced by Dr. P.K. Qasba's group (CRGSCN 16: 164-189, 1982).

3. Nuclei were isolated from mammary gland explants by homogenization buffer containing sucrose (2M), $MgCl_2$ (5mM), tris-HCL (10mM, pH 7.5), and Triton X-100 (0.2%) and then were centrifuged at 20,000g for 1 hr. Transcription of the milk protein gene mRNAs were studied by pulsing nuclei with [^{32}P] uridine triphosphate. The reaction was terminated by addition of deoxyribonuclease I (10 g/ml) and proteinase K (100 g/ml). After addition of 25g of yeast tRNA [^{32}P] RNA was isolated and hybridized to the plasmid DNA bound to DBM disks as follows:

4. Quantitation of mRNAs: the method is based on hybridization of the RNA linked to DBM-cellulose paper (filters) with a nick-translated [^{32}P] plasmid probe. RNA isolated from fresh tissue is bound to DBM-cellulose filters (usually 2 to 6 g of total RNA per 11mm circles)> The filters are prehybridized, then hybridized to [^{32}P] plasmids, extensively washed and counted. Standard curves were obtained by hybridization of known amounts of pure α-LA mRNA.

5. Agarose gel electrophoresis of RNA: estimation of the presence of mRNA in total RNA extracted from cultured cells is performed after agarose gel electrophoresis of the total RNA. RNA from the gels is transfected to DBM-cellulose paper and hybridized with nick-translated [^{32}P] plasmids. The cellulose paper is washed and then exposed to an x-ray film.

Major Findings:

A. Insulin requirement for mammary differentiation: Mammary explant cultures can be maintained viable in the presence of fetal calf serum (20%). Accumulation of milk protein mRNAs in these explants can be shown to be dependent on added insulin, hydrocortisone and prolactin. Hydrocortisone and prolactin alone do not increase 42K and 25K casein mRNAs unless exogenous insulin is added. Further details have shown that insulin dependency on the accumulation of these mRNAs cannot be replaced by multiplication stimulating activity (MSA) or epidermal growth factor (EGF). The insulin dependency can be counteracted by insulin antibody. The accumulation of the mRNAs is also dependent on the sequence of addition of these hormones and that the response can not be ascribed entirely to the hormone added

last. Pulse chase experiments with labeled uridine, in the explant cultures have shown that the influence of insulin in the presence of glucocorticoid and prolactin is on the synthesis of 25K casein mRNA. Results suggest prolactin is mainly involved in the stability of 25K mRNA, whereas insulin has little effect on the stability of this mRNA. However, in the presence of insulin the net synthesis of ths mRNA increases, suggesting that this hormone influences the transcription of 25K casein mRNA.

B. Absolute requirements of hydrocortisone for casein mRNA accumulation: 1) Accumulation of 42K casein mRNA in mammary explants from adrenalectomized, virgin rat was almost 20x higher in the presence of exogenous hydrocortisone than in its absence. 2) Accumulation of 25K casein mRNA was totally dependent on steroid. In the mammary gland from adrenalectomized virgin rat, 25K mRNA was undetectable and hydrocortisone induced this mRNA to the levels comparable to 42K casein mRNA. These results indicate much greater dependency on hydrocortisone than was appreciated previously, and also shows that the dependency does not reflect any loss of cell viability in the absence of the steroid.

Significance to Biomedical Research and the Program of the Institute:

To delineate the influence of individual hormones on the expression of milk protein genes is essential in understanding the regulation of expression of differentiated function in normal and neoplastic mammary cells.

Proposed Course of Research:

To extend these results on α-lactalbumin and whey phosphorprotein genes.

Publications:

Kulski, J. K., Topper, Y. J., Chomczynski, P., and Qasba, P. K.: An essential role for glucocorticoid in casein gene expression in rat mammary gland explants. Biochem. Biophys. Res. Comm. 114: 380-387, 1983.

Kulski, J. K., Nicholas, K. R., Topper, Y. J. and Qasba, P. K.: Essentiality of insulin and prolactin for accumulation of rat casein mRNAs. Biochem. Biophys. Res. Comm. 116: 994-999, 1983.

Chomczynski, P., Qasba, P. K. and Topper, Y. J.: Essential role of insulin in transcription of the rat 25000 molecular weight casein gene. Science 226: 1326-1328, 1984.

Sankaran, L., Qasba, P. K. and Topper, Y. J.: Effects of estrogen-depletion on rat casein gene expression. Biochem. Biophys. Res. Comm. 125: 682-689, 1984.

Topper, Y. S., Sankaran, L., Chomczynski, P., Prosser, C. and Qasba, P. K.: Three stages of responsiveness to hormones in the mammary cell. New York Academy of Science (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08212-11 OD

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
From Gene to Protein: Structure Function and Control in Eukaryotic Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Shelby L. Berger	Research Chemist	OD DCBD NCI
Other:	William H. Eschenfeldt	Senior Staff Fellow	OD DCBD NCI
	Marc Krug	Staff Fellow	OD DCBD NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
OD, DCBD

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

A/B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Methods have been developed for efficient characterization and cloning of rare messenger RNAs. The techniques include: the synthesis of double stranded cDNA; the covalent attachment of homopolymer "tails"; the measurement, in picamoles, of the amount of cDNA produced; and the measurement of the polyadenylated mRNA, in picamoles, in impure samples.

Methods have also been devised to quantify [^{32}P]-labeled macromolecules. These methods consume submicroliter amounts of material and yield highly precise measurements. They make use of Cerenkov radiation.

A cDNA library highly enriched for a specific class of T-cell products has been synthesized and screened. Approximately a dozen clones which appear to code for a lymphokine that is not fully characterized have been identified.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

Upon stimulation with mitogens or antigens, T lymphocytes from the peripheral circulation undergo reversible transformation into active cohorts of cells differing from one another in function, membrane characteristics, and the ability to elaborate soluble factors known as lymphokines. Although some of these changes have been documented biologically, the molecular mechanisms underlying the control of T lymphocyte activation have been inadequately described. In this study, we hope to understand the diversity of T lymphocyte subpopulations as well as the methods used by the cells to communicate with one another during the activation process. We will focus on nonabundant mRNAs. Reasoning that the proteins and, therefore, the mRNAs that distinguish one type of T cell from another will be rare in the general population, although perhaps abundant in a very small clone of cells, we plan to develop methods for isolating cDNA clones corresponding only to rare mRNAs. These clones will serve as probes for identifying, and later characterizing products unique to specific classes of lymphocytes. Our ultimate goal is to elucidate the function of these cells at the molecular level and to understand the cellular interactions which make possible the orderly, programmed, phenotypic transformation of T cells.

Methods Employed and Major Findings:

The isolation, characterization and amplification via cloning of lymphocyte-specific mRNAs pose technical difficulties imposed by the rarity of the mRNAs of interest, the scarcity of primary human cells and the heterogeneity of the cell population from which a specific mRNA must be obtained.

We have developed new methods for analyzing mRNA in order to conserve precious materials. These methods supercede those we have devised previously; artifacts which derive from the use of commercially prepared enzymes and sterile glass- and plasticware that are not necessarily ribonuclease free have been eliminated.

A method for determining the molar concentration of polyadenylated mRNA in solutions containing RNA contaminants was designed. Simultaneously, the level of impurities, almost exclusively rRNA in samples selected either by chromatography in columns of oligo(dT)-cellulose or poly(U) Sepharose, can be ascertained. Low molecular weight contaminants, which can easily result in the gross overestimation of the RNA concentration when absorbance measurements are used, have no effect. It should be emphasized, however, that the method is not simply an exquisitely sensitive substitute for absorbance at 260nm; instead, the method yields molar concentrations of poly(A)RNA in impure solutions rather than producing a value in μg/ml which pertains to the entire content of RNA.

The technique is as follows: An unknown amount of impure mRNA was labeled at its 3'-end with [α-^{32}P]ATP and poly(A) polymerase. The enzyme is processive, but at high concentrations in the presence of limiting ATP, only one or a few adenylic acid residues were covalently linked to the 3'-hydroxyl termini of all molecules larger than tetramers. The enzyme displayed no preference for either

polyadenylated molecules or rRNA over a broad range of concentrations, hence label was incorporated in proportion to the number of termini regardless of the nature of the mixture. Upon determining the amount of radioactive ATP incorporated, usually by assessing the amount of trichloroacetic acid insoluble material, the entire population was subjected to treatment with ribonuclease H in the presence of dT_{12-18}. Ribonuclease H specifically cleaves only RNA sequences participating in double stranded structures containing one DNA and one RNA chain. The hybrid must have a minimum of 4 consecutive base pairs to act as a substate. Thus, the adenylate residues added at to the 3'-ends of rRNA were not substrates for RNase H. After treatment with RNase H, the trichloroacetic acid-insoluble component was again assayed. Those molecules that possessed poly(A) "tails" before reacting with labeled ATP were deadenylated; label originally incorporated at the 3'-termini was released into the acid-soluble pool. All other molecules survived as part of the acid-insoluble component. The ratio of the acid insoluble radioactivity after deadenylation with RNase H to that before RNase H treatment is a measure of the percent in moles of rRNA contaminating the preparation. Since it was the rRNA and other contaminants that survived deadenylation, the method is highly sensitive to the existence of nonpolyadenylated molecules. Oligo(A) stretches in rRNA pose no problem; cleavage of rRNA at positions located far from the 3'-labeled ends produce no acid-soluble labeled fragments.

Absolute concentratons of mRNA were subseqeuntly determined by adding a known quantity of rRNA to an identical sample. Once the new percentage of rRNA, and by subtraction, the percentage of polyadenylated mRNA had been evaluated, it was possible to calculate the molar concentration of mRNA in the original solution. These measurements will be particularly useful when the composition of mRNA from unrelated cells will be compared. Clearly, the accuracy of any measurement of the abundance of specific sequences able to hybridize to a particular DNA probe is completely dependent on knowledge of the amount of mRNA tested.

Although the method worked well much of the time, there were occasions when end-labeled rRNA did not survive treatment with RNase H. In some cases the loss of radioactivity from 3'-ends was not dependent on dT_{12-18} and was not mediated by the enzyme. Apparently exonucleolytic cleavage of the RNA had occurred. A systematic survey of each element of the procedure revealed that no one substance was the cause of the problem. There were multiple artifacts operating in concert and separately. The result was an overestimate in the percent of poly(A)RNA in any preparation. Since it was important for us to have a method that always succeeded regardless of the source of commerical enzymes, and since these products are not assayed for 3'-exonuclease activity and not necessarily supplied at the stated concentration, in units of activity per microliter, the method had to be completely redesigned.

In order to neutralize both the effect of exonucleases in either poly(A) polymerase or RNase H preparations and the effect of nucleases introduced accidentally, we optimized the enzymatic reactions. By judicial manipulation of substrates and by chelation of trace amounts of metal, the rate of labeling of RNA with poly(A) polymerase and the rate of deadenylation with RNase H were increased more than 2-fold. These modifications had no effect on the contaminants. We

did not eliminate the nucleases, we simply outran them by completing the desired reactions before the undesired ones had taken place to a measurable degree.

We also found that ribonucleoside-vanadyl complexes, known ribonuclease inhibitors, could be added after labeling was completed. The RNase H activity was virtually unaffected, but some contaminating activities were abolished.

The concentration of poly(A) polymerase used to introduce radioactivity at the 3'-termini was also an important factor. Although the units of enzyme activity in the reaction were maintained at a reasonably constant level, we had no way of knowing whether we had purchased a small number of fully active, potent, enzyme molecules or a rather higher concentration of an enzyme preparation all the molecules of which had undergone some loss in activity. Since (poly)A polymerase behaves processively, the former situation could result in the polymerization of poly(A) tails on the 3'-ends of a few molecules some of which would be rRNA. Such rRNA molecules would behave as legitimate mRNA; they would be deadenylated during subsequent incubation with RNase H and thymidylate oligomers. A search for polyadenylated rRNA was undertaken and rRNA molecules with poly(A) tails long enough to be detected electrophoretically were identified. Clearly, polyadenylation had to be abolished while preserving reactions in which a few adenylate residues were added to a random distribution of 3'-termini. One possibility was to determine the specific activities of commercially available poly(A) polymerase preparations in order to avoid those which were highly active. Such an approach was not feasible; all sources were much too crude for specific activity measurements to be meaningful. A second possibility was to use higher amounts of the enzyme, but this was untenable because the glycerol in which it is supplied was inhibitory and the contaminants would also be increased. A third approach was to purify the enzyme. This project was ruled out not only because it would be time consuming but also because it would make the methodology inaccessible to all those without purified enzyme. The problem was solved with cordycepin triphosphate. Cordycepin, also known as 3'-deoxyadenosine, when phosphorylated to form the triphosphate is a chain terminator in poly(A) polymerase reactions. The 3'-hydroxyl group required for polymer formation is absent. By estimating the K_m values for both cordycepin triphosphate and ATP it was possible to formulate a mixture of substrates such that about 1 residue in 4, covalently attached to the macromolecular substrate, was a cordycepin residue. Then, regardless of the ATP concentration, there was no polyadenylation. Furthermore, using this technique, we were able to incorporate label almost as efficiently as we had done in the absence of the cordycepin derivative. If polyadenylation had been minimized by reducing the [^{32}P]ATP concentration, or alternatively, using labeled cordycepin triphosphate as the substrate, the amount of radioactivity incorporated into RNA would have been about 10-fold lower than we observed, and as a consequence the amount of RNA needed for the assay would have been 10-fold higher than that now required.

The method was streamlined by linking the two steps. Previously, poly(A) polymerase had to be removed with proteinase K which in turn was excluded by means of a phenol extraction before undertaking the deadenylation reaction. By adding vanadyl complexes to the ribonuclease H step, we were able to run the reactions consecutively without removing poly(A) polymerase and without ethanol

precipitating the labeled RNA. Ribonucleoside-vanadyl complexes served 2 roles. They greatly reduced the effect of artifactual nucleases and concomitantly inhibited both the forward and reverse reactions of poly(A) polymerase.

The results were further improved by using only sterile test tubes freshly treated with diethylpyrocarbonate to inactivate nucleases. Apparently, at the extremely low concentrations of RNA with which we work, airborne nucleases are significant.

We have developed a method for determining the molar concentration of mRNA and the moles percent contamination with rRNA with as little as 50 nanograms of material. The assay takes about 45 min to carry out. There is an error of about 10% associated with it. The method is unique. For example, ^{3}H poly(U) hybridization, a technique often used to assess the amount of polyadenylated RNA in impure samples, actually measures the amount of adenylate found in homopolymer stretches. Only with an independent measurement of the RNA concentration, heretofore available in µg/ml, an estimate of the average size of the mRNA and the expected length of the poly(A) tails, can the data from such an experiment be interpreted. Our technique eliminates the need for assumptions and provides an answer in 1/4 of the time with about 1/10 the material.

A lymphocyte specific mRNA has also been investigated. The mRNA can be induced by mitogens and appears one or two days after inactivation. It was of interest because it apparently coded for a secreted product and because sequences homologous with one of the interferons were identified at the 3'-end. The 5'-end differed from all known interferons. Hybridization experiments performed on lymphocyte mRNA fractionated electrophoretically on agarose gels and subsequently blotted onto nitrocellulose indicated that approximately 1 mRNA molecule in 500,000 coded for the desired product.

A cDNA library was prepared using unfractionated lymphocyte mRNA from cells induced with Staphylococcal enterotoxin A. The synthesis of the first strand of cDNA was carried out with a specific double stranded, denatured fragment as a primer for reverse transcriptase. Both the concentration of enzyme (1000 units/ml) and the concentration of triphosphates (1 mM, each) were adjusted to maximize the polymerase activity at the expense of the associated ribonuclease H activity. A small portion of the single stranded cDNA was analyzed electrophoretically under alkaline conditions and blotted onto nitrocellulose. From a Southern blot, we were able to ascertain the size distribution of the primer extension products. In addition to the primer itself, we found a prominent band at the position expected for the full length cDNA of interest, a band corresponding to interferon and a third band which was almost twice the size of the primer. Much later, during careful scrutiny of the cloned molecules, we were able to infer that these double sized primer molecules were self complementary hair pins. The second cDNA strand was synthesized by three enzymes acting together. Polymerase I was used essentially to nick translate the second DNA strand into existence. RNA fragments remaining bound to DNA after first strand synthesis acted as primers for a reaction that both filled in gaps and replaced RNA with

DNA. Ribonuclease H cleaved the adherent mRNA, creating nicks for polymerase I in addition to those generated during first strand synthesis by the associated ribonuclease H of reverse transcriptase. The DPN dependent DNA ligase from Escherichia coli ligated any remaining nicks by joining only DNA ends. This enzyme eschews substrates containing ribonucleotides at the terminus and performs blunt end ligations extremely inefficiently. In test reactions with globin mRNA the yield of full length double stranded cDNA was upwards of 80%. Approximately 5% or less were hairpins in which despite the presence of RNA fragments bound to single stranded cDNA, the 3'-terminus looped back and hybridized to itself creating an unwanted primer for the synthesis of the second strand.

The formation of double stranded hairpins during second strand synthesis of cDNA prevents such molecules from participating in the construction of recombinant molecules. We discovered that cDNA generated from lymphocyte mRNA contained greater than 80% hairpins. In a heterogeneous mixture, such molecules were not readily apparent when only the size distribution was analyzed. We were able to detect them in experiments designed to measure the reactivity of the 3'-ends of the newly synthesized DNA. Hairpins were recognized because only a single reactive 3'-end, that on the sense strand could be discerned. The reason for abberant behavior on the part of an entire population of molecules rather than on the part of a few molecules whose sequence and structure might favor loop formation remains to be elucidated. Hairpins were removed by treatment with S_1 nuclease.

The completed double stranded molecules were fractionated by gel filtration to remove small pieces, and tailed. It should be noted that these small pieces were undesirable byproducts of the previous reactions; in molar quantity they represented a large excess of material. If hairpins had not been eliminated, the byproducts would have been preferentially tailed and cloned. The final library would have been devoid of large inserts.

The covalent linkage of homopolymeric deoxyguanylate tails to the 3'-ends of double stranded cDNA served 2 functions. It not ony allowed the insert to anneal to linear plasmid molecules with deoxycytidylate tails, but also it made possible the determination of the number of cDNA molecules in the reaction. Because we discovered that the length of dG-tails can be rigorously controlled independently of the DNA concentration, we were able to use the incorporation of label from [^{32}P]dGTP into 3'-termini as a measure of the number of termini in the reaction. Knowledge of this value made the choice of vector concentration for efficient cloning a simple matter.

After transformation a cDNA libary consisting of 6000 to 8000 clones was obtained and replicated onto nitrocellulose filters. The library was evaluated in several ways. A random sample of clones was selected and grown up. Upon electrophoretic analysis, only large DNA inserts were found in all cases. Clones from one filter capable of hybridizing with the primer were also examined. Out of 12 of these, all contained inserts of primer molecules which had not been extended. Give the incidence of the desired mRNA in the bulk population, the size distribution of the antisense, single stranded primer specific cDNA, the concentration of primer, and its size, a large number of clones containing only

the primer were anticipated.

The cDNA library was also screened with a sequence obtained from a human gene library cloned in lambda Charon 4a. The genomic location of the 5'-end of the lymphocyte specific mRNA under consideration was identified by examining a selected portion of the human genomic library using as a probe, labeled, gel-purified, full length cDNA. Clearly, this cDNA represented a very precious subset of material similar to that comprising the Southern blot. The cDNA library contained approximately 500 clones which were positive for the genomic fragment from which the 5'-end of the mRNA was believed to be derived. Since 48 of these chosen at random did not contain sequences homologous with the primer, further information was needed to target interesting clones.

The information was obtained by screening the entire library with the primer. Although approximately 1000 colonies were found by this technique, only 61 of them appeared to be positive for the 5'-segment as well. On careful rescreening, all 61 putative double positives turned out to be mixtures of different recombinant bacteria the individual members of which were positive for only one sequence. Apparently there were to be no shortcuts.

We examined the thousand clones which hybridized to the primer by dividing them into groups of approximately 6 clones each. DNA from the mixtures was prepared and analyzed electrophoretically. Since these recombinants were chosen from dense areas with many colonies crowded together on a plate, many of the individuals were not primer positive. Consequently the electrophoretically purified inserts, which reached a maximum size of greater than 2 Kb, were blotted onto nitrocellulose and rescreened with the primer. The individuals in the group that had been responsible for the initial signal were detected and isolated. From the entire pool of colonies many positives were found but only 10 to 12 colonies contained inserts that were significantly larger than the primer itself. It is among these which we hope to find the desired clone. None is full length, suggesting that the genomic sequences that we identified with full length cDNA are located near the extreme 5'-end of the cDNA.

We learned several interesting facts from our library. First, the vast majority of the clones contained DNA which was primed by molecules other than the specific DNA fragment we had included for the purpose. Among the possible rogue primers were: (1) oligo(dT)$_x$ leached from the oligo(dT)-cellulose column in which the mRNA was purified; (2) poly(A) termini; and (3) random RNA fragments generated by the associated RNase H acting on any DNA:RNA hybrids in the reaction mixture. Although RNA is not as efficient a primer as DNA, even poor priming might produce many more clones than specific priming given a low enough incidence of homologous mRNA. In order to put these results in perspective we calculated the number of recombinants anticipated from 10 µg of polyadenylated mRNA if oligo(dT)$_{12-18}$ had been the primer. Give the efficiency of each step, including transformation of E. coli with tailed, circular plasmid molecules containing annealed inserts, we estimated that 10^7 clones could have been produced. Thus, the existence of several thousand primer-independent clones, when viewed with respect to the number of clones usually seen in a typical experiment, does not seem excessive. Furthermore, given the incidence of the lymphocyte specific mRNA in question, the total amount of input mRNA and

the efficiencies referred to above, only about 20 of the desired clones are expected. This estimate compares well with the actual 12 to 13 colonies we have identified.

Significance to Biomedical Research and the Program of the Institute:

This project conforms to Objective #3, Approach #1 of the National Cancer Plan. It aims at developing methods that can be used to understand the biochemical control mechanisms by which normal cell growth and function are maintained. Disordered cell growth in neoplastic populatins may then be better understood and rational attempts made to prevent or to modify it.

Proposed Course of the Project:

Further studies will continue to focus on developing efficient methods for cloning and screening cDNA and/or gene libraries. These methods will be applied to the study of rare mRNAs found in activated T cells.

Publications:

Berger, S.L., and Krug, M.S.: A simple method or quantifying ^{32}P in submicroliter samples. Biotechniques 3: 38-46, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05526-17 OD

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
P53: A common protein in embryonic differentiation and in cellular transformation.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Peter T. Mora	Supervisory Chemist	OD DCBD NCI
Other:	V. W. McFarland	Chemist	OD DCBD NCI
	Usha S. Thathamangalan	Visiting Fellow	OD DCBD NCI
	C. Dale Smith	Visiting Fellow	OD DCBD NCI

COOPERATING UNITS *(if any)*

Daniel Simmons, University of Delaware; Janice Chou, MGB, NICHHD, NIH

LAB/BRANCH
OD, DCBD

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The complexing of the p53 to the SV40 T antigen was studied in various cells. All p53 is in complex in almost all SV40 transformed murine fibroblast cells, however, in SV40 transformed Balb 3T12 cells most (>80%) of the p53 is free. This difference is not due to differences in the primary sequence of these two proteins, but correlates with a five times lower phosphorylation of the non-complexed protein species. SV40 transformed "immortalized" human placenta and amnion cells, which are epthelial and of throphoblast origin, contain only non-complexed p53. These findings pose the question to what extent, if any, the complexing with p53 is necessary for the transforming functions of the T antigen.

In the developing mouse embryo during the second half of gestation the steady state level of p53 declines drastically, however, the p53 mRNA level seem to remain constant. This is in contrast to what occurs in cells stimulated to divide in culture, when there is a coordinated modulation of p53 and mRNA levels during the cell division cycle. The apparent translational modulation of p53 in embryogenesis is under study.

We are working on a large family of highly tumorigenic mouse cell lines and clones ($TD_{50}=10^2$) selected out from near normal ($TD_{50}=10^{6.4}$) parental cloned fibroblasts by a single transplantation step. A very complex and interesting picture of transcriptional modulation is now emerging for numerous proto-oncogenes (and also for p53), but none which is qualitatively different between the normal parents and all the (mutant) tumorigenic progenies. The system is very useful to study the multifarious molecular possibilities which can be associated with the tumorigenic transformation. The levels of p53 is the same in the normal cells and in the tumorigenic derivative cells.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

There is increasing evidence which shows the importance of the cellular protein p53 in the metabolism and the growth of normal mammalian cells, especially when such cells are rapidly dividing and are undifferentiated. We focused our work to study p53 biosynthesis as it relates to cell growth and embryonic differentiation. In addition we carried out studies to define to what extent the complexing of the p53 with the SV40 T antigen, or the elevated amount of p53 in certain transformed cells may pertain to cellular tumorigenicity. We also investigated a family of spontaneously transformed (mutant) cells for signs of increased ex-expression of many proto-oncogenes.

Methods Employed:

Labeling of proteins and nucleic acids with radioactive precursors during cell growth in culture. Fractionation techniques and isolation of (labeled) subcellular components and of proteins and nucleic acids. Isolation and characterization procedures for biologic macromolecules: thin-layer and column chromatography, gel electrophoresis, electrofocussing, and autoradiography of gel slabs, a variety of nucleic acid transfer hybridization techniques, analytical and preparative ultracentrifugation studies. Complex immunochemical techniques using monoclonal antibodies for identification of antigens, for preparative work and also for quantitation such as in Western blots.

Major Findings:

1. Complexing of the p53 to the SV40 T antigen. Originally all of the p53 has been found to be in complex with the T antigen in numerous SV40 transformed murine fibroblast cells which have been investigated. As the T antigen is responsible, inter alia, for the initiation and maintenance of transformed cell growth phenotype in tissue culture, this complexing was thought to be important, if not essential for the transforming function of the SV40 antigen. This observation, together with elevated levels of p53 having been observed in cells transformed by other diverse means, stimulated much research on p53 in the last five years. At the start of this work, our laboratory was the first to observe and report that a cellular DNA encoded protein, now referred to as p53, occurs in elevated amounts in many SV40 transformed murine fibroblast cells. We were able to do this, in part, because of the large selection of murine fibroblast cells and their SV40 transformed derivatives we prepared and had available for comparative biochemical and biological studies. Recently, however, we found that a distinct family of mouse fibroblasts, all from the Balb/c 3T12 cells, after SV40 transformation contains p53 substantially all in a free non-complexed form. These experiments were carried out by measuring metabolically labelled p53 and T antigen. We now also measured the steady state levels of these proteins in Western blotting using appropriate monoclonal antibodies, and compared the complexed and non-complexed amounts of these two proteins, and confirmed that all SV40 transformed derivatives of Balb 3T12 cells possess virtually only non-complexed p53. When trying to account for this, at first we could not find easily detectable structural differences such as in

comparing ^{35}S-methionine labelled tryptic peptides of both p53 and T antigen, of both the complexed and non-complexed variety. To fully exclude the possibility that a structural change in the primary amino acid sequence (mutation) in the T antigen could account for the non-complexing, we rescued the SV40 virus from an SV40 transformed Balb 3T12 cell. This rescued virus was used then to transform another mouse cell, the AL/N 210C clone which from earlier experiments was known to provide 100% complexed p53 after transformation with wild type SV40. Similar results were obtained after transformation with the rescued virus. This experiment conclusively proved that change (mutation) in the SV40 T antigen region cannot be the cause of the non-complexing.

We found one close, and up to now binding, biochemical correlate of the non-complexing: there is a greatly (5X) reduced phosphorylation in both of the p53 and of the T antigen when there is no complexing, as compared to when there is full complexing. Phosphorylation is a post-translational modification of many proteins which often correlates with differences in protein functions, and generally reflect crucial processes in cell biology and biochemistry, often pertinent to cell division control. We have started a comprehensive study of phosphorylation of p53 and T antigen, in collaboration with the laboratory of W. Fiers in Belgium, using numerous cell families developed in our laboratory and elsewhere, and we are comparing biochemical data with observation on anchorage independent cell growth in culture and also with cellular tumorigenicity in vivo.

Other investigators recently published that: 1) SV40 transformation of murine fibroblasts for differential growth phenotypes in culture can also occur with SV40 early gene deletion mutants, which produce truncated T antigen which then does not complex with the p53; 2) The complex formation is not cold sensitive in cold sensitive transformant cells induced by a SV40 ts A mutant; 3) Fully transformed cells can be derived from transfection with adeno DNA fragments which do not possess the coding region for the p58 adeno protein, which is the adeno virus early gene product previously observed to complex to p53. These observations, together with our findings summarized above, now indicate that the complexing between the SV40 (and adeno) early viral gene products and of p53 is not involved in the initiation and maintenance of transformed growth phenotype of fibroblast cells in culture. We also showed that similar conclusion applies to cellular tumorigenicity in vivo.

It is important to note that all the above discussed studies were on murine fibroblasts, or on sarcoma producing cells. With Janice Chou, NICHHD, we extended these studies to normal epithelial cells of human origin. We found that in human fetal placenta cells, both from first trimester pregnancies and also at term, when immortalized by transformation with (ts A mutant) SV40 there is only non-complexed p53 and T antigen (at both permissive and non-permissive temperatures). Similarly, there is preliminary evidence that there is almost complete absence of complexing in term human anion cells transformed by origin minus and tsA mutant SV40, when cells are grown at the permissive temperature and synthesize normal T antigen. All these epithelial cells arise from the embryo throphoblast.

The term human placenta appears to be a good source to obtain a large quantity of non-complexed p53 with the appropriate post-translational modification(s). A collaborative work is being set up with a clinical investigator (Dr. D. Bonds, Hospital of the University of PA) who is interested in fetal and placental physiology.

2. p53 in embryogenesis and in cell growth. p53 also occurs in normal embryo cells, but generally it has a short half life and it is difficult to detect and quantitate. Nevertheless, we were able to show before that the metabolically labelled p53 rapidly declines during the embryonal development of the mouse in the second half of gestation. Now we measured the steady state levels of p53 and also of p53 mRNA (by Western and Northern blots, respectively) in different stages of embryonal development in whole mouse embryos, and also of cells in primary culture, and compared these with levels in cultured mouse embryo fibroblasts clonal cells, and in a multipotential embryonal carcinoma cell line (F9). In all cases the p53 mRNA (as detected by a p53 cDNA probe) appears as a single band, corresponding to 18S or 2kb size. In the differentiating mouse embryo during the second half of gestation we found 10 fold decrese in the p53 levels, but apparently no decrease in the p53 mRNA levels: It seems that post-transcriptional control(s) operate which vary the p53 levels in the normal process of differentiation. These controls seem to be different from those which appear to function in cells stimulated for example by serum to reenter the cell growth cycle in culture: it was observed by others that there is a simultaneous increase in both p53 and p53 mRNA in the late G1 phase of the cell cycle.

3. Cell immortalization, tumorigenicity and the expression of p53 and of known proto-oncogenes. When cells are transfected with DNA constructs containing activated p53 cDNA, certain cells are immortalized, and when co-infected with cRas DNA, they acquire ability to grow in tissue culture without anchorage and to form tumors in vivo. This recent observation by others placed p53 in the category of potential proto-oncogenes, similar to c-myc. We have available families of closely related, well pedigreed and biologically characterized mouse embyro fibroblast cell lines for comparative studies. These cells all originated from normal or low tumorigenic lines and clones, such as the well studied AL/N 210C clone ($TD_{50}=10^{6.4}$ cell/mouse) and the 216C clone ($TD_{50}=10^{5}$). A large number of these cells (cf 10^{7} or 10^{5}), necessary for the induction of tumors, were injected into the syngeneic mice, and the tumors were reestablished as tumor lines in culture. All 12 tumor lines had very high cellular tumorigenicity ($TD_{50}=10^{2}$), and several clonal derivatives of three tumor lines tested maintained this tumorigeneic phenotype. Apparently the transplantation gave preferential growth to, and selected out from the normal cloned cell population, a (few) mutant cell(s) which acquired tumorigenicity spontaneously. These laboratory obtained tumors are probably as close to human (spontaneous) tumors in vivo as one can have in a cellular model suitable for study. Several tumor lines and tumor cell clones were then compared with their normal non-tumorigenic parent clones for altered expression of the most frequently studied cellular proto-oncogenes and also of p53. For control the embryonal carcinoma cell line F9 was also tested. Poly A^{+} RNA isolated from these cells were electrophoresed under denaturing conditions, and then transferred to and immobilized on nitro-cellulose membranes prior to probe hybridization under stringent conditions. Employed were labelled v-onc probes: Ha-ras, Ki-ras, myc, raf, fos, abl, src and myb; a p53 cDNA clone was also used.

With the exception of myb, all probes detected bands, some clearly multiple bands: cf., Ki-ras 2 bands (1.6 and 3.0 kb), myc 3-4 bands (2-3 kb range), essentially in all the cells tested. The relative intensities of the bands varied from cell line to cell line and several interesting leads have appeared Current experiments are in progress with c-onc gene probes. We are also testing the cells for possible mutated c-ras oncogene (in collaboration with M. Barbacid).

Up to now expression (or co-expression) of several of the best studied putative proto-oncogenes (and of p53) tested did not show a general (binding) correlation with the appearance of cellular tumorigenicity and of anchorage independent cell growth in all the tumor cell clones. However, much other useful novel information already has been obtained, such as (normal) expression in normal fibroblast clones and in cloned tumor cells of several of the cellular proto-oncogenes and of p53 in a reproducible manner. This model system now allows interpretable studies on cloned cells and their descendants of known pedigree and of great differences in tumorigenicity, when the tumorigenicity originated spontaneously in (a) very rare cell(s), apparently by mutation(s), and thereby is useful to test many other and potentially very diverse molecular correlates of the apparently multiple steps in oncogenesis in vivo, beside the dominant effects of hitherto recognized proto-oncogenes now usually studied.

Significance to Biomedical Research and to the Program of the Institute: Proteins, such as p53, which are involved in the metabolism and the growth of cells especially when the cells are rapidly dividing and are undifferentiated such as in embryogenesis, are of fundamental importance to cell biology. It is also important to carefully and critically define the potential involvement of such proteins in cellular transformations and in abnormal growth.

Proposed Course of the Project:

Several plans and ongoing projects are already mentioned above. These include studies on phosphorylation of p53, nature of p53 in throphoblast derived embryonic cells including the placenta and the ammionic cells, regulation of p53 expression in embryogenesis vs in cell division cycle, etc. Studies on changes on p53 processing in early stages of embryogenesis with in situ detection of mRNA are planned to be set up with H. Westphal, NICHHD. The family of spontaneously transformed cells will be further studied as implied above.

Publications:

Mahavedan, V., Chandrasekaran, K., McFarland, V. W., Simmons, D. T., and Mora, P. T.: The p53 is not necessarily bound to the T antigen in SV40-transformed mouse cells. Mol. Cell. Biology (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00941-29 OD

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic and other factors affecting marrow transplantation in irradiated inbred mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Delta E. Uphoff Research Biologist OD DCBD NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
OD, DCBD

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B/D

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The investigation of physical factors affecting the success of bone marrow transplantation experiments was continued and include both radiobiological effects of the direction of the exposures and subtle changes in physical factors that alter the survival of irradiated inbred mice with and without marrow graft. Basic concepts of radiation biology were demonstrated to be invalid and physical factors, considered of little consequence when applied to biological systems, were critical for the reproducibility of these experiments. For example exposure-rate effects attributable to cell proliferation and repair during protracted and fractionated exposures also occurred over short exposure times as a result of repair of sublethal radiation damage, i.e., identical results were obtained when simultaneous 2 directional exposures were administered over the same time span as the dorsal exposure. In addition sequential alternate exposures were less effective than either two direction or unidirectional exposures when treating the circulating and fixed cells of the hematopoietic system. X-rays are of different wave lengths and the "quality" of the X-rays have been defined in terms of the filtration by copper and aluminum and the half value layer. These crude methods of "definiting" the quality of X-rays have been demonstrated to be inadequate. New more precise methods of determining these parameters must be found if experimental conditions are to be met and experimental data reproduced.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

To investigate genetic and physical factors influencing the success of bone marrow replacement following administration of toxic levels of ionizing irradiation and/or chemotherapeutic agents used for cancer treatment. Although the ultimate goal was to devise methods of preventing and controlling lethal graft-versus-host reactions while utilizing immunotherapeutic benefits of less severe reactions, it became apparent that to obtain consistently reproducible results a reevaluation of some universally accepted concepts and practices used for irradiating recipients was required.

Methods Employed:

To test the validity of radiobiological concepts as applied to hematopoietic cells and tissues required an experimental system whereby the effects of single variables could be tested. Another requirement was the involvement of intact animals since under these conditions hematopoietic cells do not constitute a fixed target. If intact animals were to be tested and the variables limited, strict control of the source of genetically defined animals was necessary. All inbred strains and substrains were raised under conventional conditions in a closed colony set up for this purpose. Foster nursing and ova-transfer techniques used to produce germ free and specific pathogen free mice introduce maternal influences which were readily demonstrable in marrow transplantation experiment. These material influences modify radiation resistance and sensitivity as well as the expression and recognition of tissue antigens. Thus all derived substrains must be traceable to a single foster mother to mimmize the variables introduced through material influences. All tests involved more than one inbred strain to eliminate the possibility that the data reflected a unique strain difference rather than a general phenomenon. Both the X-ray machine and the Gamma Cell-40 were capable of simultaneous 2 directional (dual) exposures and the unidirectional dorsal and/or ventral exposures.

Major Findings:

The first physical factor found to affect the survival of marrow inoculated mice was the "quality" of the X-rays used. The spectrum of X-rays has been defined for biological purposes in terms of filtration while the quality was defined in terms of half-value layer (HVL). These are imprecise, easily obtained measurements but considered adequate for biological purposes, since few biological systems can detect a 25R difference in exposure. However, subtle differences in X-rays not detectable by 2 different methods of calibration and not involving changes in filtration or HVL significantly altered the survival of marrow inoculated mice. These experiments involving multiple mouse strains were alternately reproducible using either the Westinghouse or Philips 250 KV X-ray machines. Shifts in survival also were revealed when comparing data obtained with the 2 X-ray machine even though the same filters were used and the Philips machine was operated at 235 KV to produce

the same HVL as that of the Westinghouse equipment. Determining the spectrum of the wavelengths of an X-ray beam is apparently a difficult problem which has not yet been resolved. However, the wave form of the current has been determined under the same conditions that altered the survival of the mice. For example, the difference in wave form between X-rays produced with and without the auxilliary line voltage regulator was an increase in amplitude when the voltage regulator was added. The significance of this measurement has not been fully evaluated since increasing the amplitude would be expected to change the calibration but no change in calibration was detectable. One possible approach would be to find a method to indirectly demonstrate changes in the quality of the X-rays without actually measuring the critical wave length(s) involved.

Investigations of directional affects of exposure to X-rays resulted from the observation that the same dorsal and dual exposures produced different results in marrow inoculated mice. Since dual exposure equipment was not universally available, a complete evaluation of varous combinations of exposure was undertaken using a less than lethal exposure but without marrow inoculations. The aim in marrow transplantation was to deplete the hematopoietic system to permit engraftment of the marrow. These data were initially reported as mortality from hematopoietic depletion. Reviewers faced with new ideas or concepts based on mortality data tend to conjur up excuses for why animals die rather than considering the plausibility of the data presented. In retrospect survival data might be more acceptable since there are fewer exotic reasons for survival of irradiated animals compared with mortality. Hematopoietic cells unlike other tissues do not constitute a fixed target, therefore, a mid-line focus used for all exposures. The reduced effectiveness of dorsal exposures was an exposure-rate effect rather than the lack of homogeneity postulated by Friedman and Lorenz in 1939 when they first described the advantages of simultaneous 2 directional exposure. To demonstrate this finding required that dual exposures be administered over the same elapsed time as dorsal exposures. This was accomplished by reducing the current which duplicated the curvers obtained with dorsal exposure. Exposure-rate effects have been attributable to cell proliferation and/or repair of radiation induced cell damage. Assuming there are only two mechanisms for the exposure-rate effect, the exposure times used were too short (10-15 min.) to expect cell proliferation. This left the alternate explanation attributing this effect to repair of sub-lethal radiation injury. The submitted report of thse observations was rejected. However, the March '85 issue of Radiation Research contained a report by Rhee et al. presenting corroborative evidence for repair of sub-lethal radiation damage in tissue cultures of human leukemia cells exposed to both X-rays and gamma radiation from Cs137 at different exposure rates and with short exposure times. These data will rewritten and submitted to another journal.

Ventral exposures were least effective for in addition to the exposure-rate effect, there was sufficient absorption of radiation to reduce the exposure to hematopoietic cells distal to the radiation source. Using thermoluminescent dosimetry (TLD) the difference between entrance and exit doses was a reduction of 34%. This was greater absorption than required to explain the data on ventral exposures. The TLD data was judged to be inaccurate by reviewers since the absorption in the lucite block was greater than in the intact animal.

However, in animals the endpoint was the effect of exposure of both fixed and circulating hematopoietic cells to X-rays over an exposure time already demonstrated to allow for possible repair of sub-lethal radiation damage. Under these conditions absorption should be less than the total absorption should be less than the total absorption measured by the fixed TLD targets in a lucite block without repair capabilities. This discussion was not included in the submitted paper which was already considered too long and complicated for most readers.

There is still no rational explanation for the enigma that sequential alternate exposures were not equivalent to each other but a function of the direction of the first exposure. Since these exposures were subject to an exposure-rate effect they would not be equivalent to dual exposures. In addition half the exposures were subject to the absorption phenomenon of the ventral exposure and would not be equivalent to a fully dorsal exposure. Therefore sequential alternate exposures of circulating targets would have the disadvantage over dorsal exposures for total body irradiation since they would require an increased exposure to obtain the same effect on the hematopoietic system. Whether the difference between sequential dorsal + ventral and ventral + dorsal exposures would increase with the size of the animal would depend upon the mechanism involved. However, the unequivocal results obtained with mice would influence the success of the marrow grafts if the same exposures were administered over the same time interval as the dorsal exposure, i.e., alternate exposures would result in fewer successful engraftments and/or a greater reversal to host genotype.

In the investigation to determine the time required to neutralize the radioprotective effects of nembutal observed between 15 min.- 60 min. after anesthesia, additional data established the existence of a rebound effect occurring after 90 minutes and lasting 120 min. or longer. It was concluded that protocols requiring anesthesia before or during irradiation should also require that a fixed time be used between the inoculation of the nembutal and the initiation of the irradiation if reproducible results are to be obtained. These experiments were a spin-off of a test to determine whether irradiation from either the upper or lower tube would produce identical results, i.e., were directional effects a function of the quality of the radiation from 2 different tubes? A previous report combining the directional effect and the effects of anesthesia was rejected. Data on the effects of anesthesia alone were extracted and presented in a brief, straight forward report on the results of irradiating mice at different times after anesthesia. This report was rejected by reviewers for lack of discussion of the mechanism for the protective effect and information on the number of stem cells in cycle or in the resting state with reference to their relative radiation sensitivity. When initiated these were simple experiments to determine the length of time required to neutralize the radioprotective effect of anesthesia. They were not designed to test the effects of hypoxia, temperature reduction or any other conditions affecting the survival of anesthetized animals nor were we equipped to investigate cells cycling in intact animals. However, the existence of the protective effect long after the mice were awake and the observed rebound effect would impact on many types of experimental procedures. There is no way to estimate the time and money already

wasted by conducting experiments in which the time interval between anesthesia and irradiation was an unrecognized variable. In time these data will be submitted for publication to another appropriate journal.

Experiments involving directional effects of gamma radiation are in progress. The higher energy gamma radiation has greater penetration with less absorption. This results in a higher lethal exposure and a possibility of variations resulting from the size of the mice. Variability was observed and effected primarily the comparative results of dual and dorsal exposures. At the exposures used for gamma irradiation the difference beween dual and dorsal exposures was less evident than with X-rays. The effectiveness of ventral exposures was still greatly reduced. Since dose-rate effect using Cs137 was reported by Rhee et al. in the tissue culture system, the nature of the variation observed in intact mice requires further investigation. Initially some variability may have been was introduced by not aging the mice as long before exposure as had been our usual practice with X-rays in an effort to save animal room space. All exposures are again being administered at 12 weeks of age. One method of testing for an exposure-rate effect in mice would require use of attenuators which could alter the quality of gamma irradiation as a result of scatter. In preliminary experiments sequential alternate exposures are once again not equivalent to each other. It will be necessary to make further adjustments in the exposure in order to obtain more definitive results on directional and exposure-rate effects.

Significance to Biomedical Research and the Program of the Institute:
Initially the field of experimental marrow transplantation developed rapidly with investigators using available equipment and whatever protocol produced success in their experimental animals. There was little if any effort to standardize the treatment. Similarly clinic treatment of malignant and nonmalignant hematopoietic disorders has increased in the absence of any general agreement on the protocol for total-body irradiation. Interest in the clinical use of bone marrow transplantation is expected to continue and be expanded to include an increasing number of matched unrelated donors for patients for whom no other effective treatment exists. Although total-body irradiation is used to prepare a select few recipients for marrow grafts, the lack of a standard protocol may contribute to the varying degree of success experienced by different treatment centers. Until critical factors are specifically defined there will continue to be little if any standardization of treatment. Unlike chemistry and physics, biology is not an "exact" or predictable science but a complex interaction of many systems. In addition, irradiation of the hematopoietic system consisting of circulating cells and fixed tissues, presents unique problems which can only be resolved by experiments using intact animals. Generalization established 50 years ago are doggedly being pertetuated e.g., the supposition that biological systems are too insensitive to detect qualitative differences in X-rays has persisted as "fact" by repetitition rather than by surviving scientific challenge. Experimental marrow transplantation has been demonstrated to be a particularly sensitive system affected not only by the quality of X-rays but by directional effects of exposure, exposure-rate effects and fractionation of the exposure which have not previously been recognized as critical for successful engraftment. Progress depends upon

reproducibility of experimental data. Critical factors influencing this reproducibility must be defined and reported to stimulate interest in designing experiments that may improve results and contribute to progress rather than to perpetuate the "me too" research fashionable today. However, in recent years there have been increasing examples of blocking publication of new data in an apparent effort to maintain the status quo by peer reviewers. An opinion has been expressed by some scientists that reviewers facing with the realization that their experiments may be based upon incorrect or inadequate premises may reject papers in fear of facing reduced financial support if new or conflicting data are published.

If this speculation proves correct, it is a sad commentary on modern education and the integrity of todays scientists. However, much of the responsibility for these conditions rest with management and the requirement that reports of research be published in journals requiring editorial refereeing. New and interesting data should be made available for evaluation by all scientists and not left to a few self-serving referees who appear to resist progress by rejecting new data that fails to support existing concepts. Only after defining and resolving the complex problems of preparation of recipients for marrow grafts can problems of the graft-versus-host reaction and other complication be resolved with satisfaction.

Proposed Course of the Project:

First priority must be given to continued efforts to publish existing data and conduct experiments considered essential by reviewers. To accomplish this most effectively will necessitate the determination the quality of X-rays produced by the 250kV X-ray machine operated with and without the auxillary line voltage regulator. Only then will publication of the data on the effect of the quality of X-rays on the success of marrow transplantation and comparison between data obtained with the Westinghouse and Philips equipment be possible. It was this observation that demonstrated that subtle changes in the conditions used to prepare a recipient for a marrow graft could affect the success of the engraftment and/or survival. Once the sensitivity of the marrow transplantation system is established cross referencing should prove effective in supporting the validity of other data. Experiments using gamma radiation from cesium 137 will continue.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05544-15 OD

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cell Surface Changes in Transformed Mouse Cell Lines

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Samuel W. Luborsky	Chemist	OD DCBD NCI
Other:	Peter T. Mora	Supervisory Chemist	OD DCBD NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
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SECTION

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NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In work reported previously, we had found low levels of radioactivity associated with the immunoprecipitates (IP) obtained by treatment of extracts of ^{3}H-uridine labelled SV40 transformed mouse embryo cells with hamster anti-T antigen serum (T sample). Such radioactivity was not observed in samples obtained following treatment of such extracts with buffer (control, N samples). This work was repeated. Although more tissue culture flasks of cells were used in each experiment, essentially the same results were obtained, even after extensive washing of the IP under a variety of conditions. These results appeared to confirm those obtained earlier. The levels of radioactivity observed were low, however, and presented the necessity of distinguishing between contamination of the IP by unincorporated ^{3}H-uridine (artifact), or low levels of RNA synthesis (real incorporation).

Project Description

Objective:

To determine if RNA is present in immunoprecipitates of extracts from SV40 transformed mouse cells.

Methods Employed:

Tissue culture cell cultivation; cell cloning procedures; polyacrylamide gel electrophoresis; column chromatography; ultracentrifugation; immunoprecipitation and various immunological assays to detect tumor antigens.

Major Findings:

In work reported previously, we had found low levels of radioactivity associated with the immunoporecipitates (IP) obained by treatment of extracts of ^{3}H-uridine labelled SV40 transformed mouse embryo cells with hamster anti-T antigen serum (T sample). Such radioactivity was not observed in samples obtained following treatment of such extracts with buffer (control, N samples). This work was repeated. Although more tissue culture flasks of cells were used in each experiment, essentially the same results were obtained, even after extensive washing of the IP under a variety of conditions. These results appeared to confirm those obtained earlier. The levels of radioactivity observed were low, however, and presented the necessity of distinguishing between contamination of the IP by unincorporated ^{3}H-uridine (artifact), and low levels of RNA synthesis (real incorporation).

Significance to Biomedical research and the Program of the Institute:

Cell components like the SV40 T antigen and the cellular p53 protein, which appear in many cell types following cell transformation by SV40, appear to influence cell properties, antigenic character, and growth characteristics. A better understanding of the properties of these components may help us better understand the mechanisms of normal and of abnormal cell growth. The reported presence of RNA in the cells is similarly of interest, broadening the types of molecules which might play a role in determining cell properties.

Proposed Course of the Project:

Because of the higher priority of other research objectives, and of technical difficulties associated with proving that such low levels of radioactivity associated with the IP are real, not simply the result of contamination of the IP with unincorporated ^{3}H-uridine, this project was terminated.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08901-1 OD

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies of Animal Cell Adhesion

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Samuel W. Luborsky	Chemist	OD DCBD NCI
Other:	Kenneth M. Yamada	Chief, Membrane Biochemistry Section	LMB DCBD NCI

COOPERATING UNITS *(if any)*

None

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SECTION

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B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cells of most vertebrate species contain proteins, such as fibronectin, whose function is to facilitate cell adhesion to extracellular substrate surfaces or to other appropriate cells. Assays reported in the literature are available to quantitate these proteins. I obtained instruction in these assays from Yamada's group, and tested various parameters of the assay procedure. I determined the optimum level of fibronectin required to coat the plastic surface of the assay wells, and the optimum incubation time required for the assay. The effect of certain fibronectin-derived peptides was tested on fibronectin-mediated BHK cell adhesion to the surface of the plastic assay wells. From such assays as these, Yamada has ranked the various peptides in order of their relative activities in promoting cell adhesion to the fibronectin and other adhesion proteins. I have also focussed on work with molecular models, to construct some of these peptides. Data for most of the bond lengths and bond angles were found in the literature. These models should help determine crucial structural features involved in cell binding and adhesion.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objective:

To analyse the molecular mechanisms of cell adhesion.

Methods Employed:

Tissue culture cell cultivation; cell cloning procedures; polyacrylamide gel electrophoresis; column chromatography; ultracentrifugation; immunoprecipitation and various immunological assays to detect tumor (and other) antigens; use of molecular models to build model peptides.

Major Findings:

Cells of most vertebrate species contain proteins whose function is to facilitate cell adhesion to extracellular-substrate surfaces, or to other appropriate cells to form tissues and organs. Anchorage-dependent cells undergo mitosis and proliferate only when firmly attached to a surface. Assays have been developed and reported in the literature to detect and quantitate these proteins, using cell spreadng on a substratum as a measure of cell adhesion. Fibronectin is one of these adhesion proteins, which provides an essential physiological bond between substratum and anchorage-dependent cell. I observed some cell adhesion assays performed by people in Yamada's group, obtained protocols, and carried out assays. Instruction was obtained in scoring cell attachment and spreading. BHK cells were grown in culture as needed for these assays. Various parameters of the assay procedure were tested, such as the optimum level of fibronectin required to coat the plastic surface of the assay wells, and the optimum incubation time required for the assay. The effect was tested of certain fibronectin-derived peptides on fibronectin-mediated BHK cell adhesion to the surface of the plastic tissue culture wells. From such assays as these, Yamada has ranked the various peptides in order of their relative activities in promoting cell adhesion to the fibronectin and other adhesion proteins. Clearly, an immediate question is what structural feature(s) the active peptides have in common, which might be different in the inactive peptides. I have, therefore, also focussed on work with molecular models, to construct some of these peptides. Data for most of the bond lengths and bond angles were found in the literature, and models are now being constructed. These models should help us to determine the crucial structural features involved in cell binding and adhesion.

Significance to Biomedical Research and the Program of the Institute:

Study of the extraction and properties of cell adhesion proteins can contribute to our better understanding of the processes involved in tissue formation and control of growth and development. Such processes should relate to the mechanisms of normal, as well as of abnormal, tissue growth and development. Invasion and metastasis are thought to involve abnormal interaction of cells with other cells and/or with cell matrices. Studies of cell interactions are therefore important to determine the mechanisms involved in metastasis.

Proposed Course of the Project:

Studies of the properties of cell adhesion proteins and of their cell receptors will continue. On the basis of the adhesion assays and rankings of a number of peptides for their ability to stimulate cell attachment to firbonectin and other adhesion proteins, the molecular structure of these peptides, as shown by the molecular models, will be examined to determine if there is any obvious difference between active and inactive peptide structures, and to attempt to correlate peptide strucutre, molecular conformation, and effect on cell attachment. If from the models we can determine crucial structural information, we might be able to predict the structure of more efficient inhibitors of cell adhesion. Such inhibitors might be useful in inhibiting tumor cell invasion and metastasis.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08902-1 OD

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Chemical Characterization of An Immunosuppressive Glycoprotein

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, leboretory, and institute affiliation)*

PI:	Sidney Shifrin	Chemist	OD DCBD NCI
Other:	Andrew V. Muchmore	Medical Officer	MET DCBD NCI
	David Zopf	Medical Officer	LP DCBD NCI
	William Coleman	Research Microbiologist	LBP NIADDK
	Akira Kobata	Fogarty Fellow	FIC

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
OD, DCBD

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to characterize the structural components of uromodulin which impose immunosuppressive properties on the urinary glycoprotein.

PHS 6040 (Rev. 1/84) GPO 914-918

Thus far, it appears that the polypeptide moiety of uromodulin is not involved in the immunosuppressive properties of this urinary glycoprotein.

Attempts were therefore made to isolate and characterize the immunologically active glycopeptide by several different methods.

(1) Removal of N-linked oligosaccharies with N-glycanase, N-Glycanase (Peptide: N-glycosidase F) was highly purified from cultures of Flavobacterium meningosepticum and hydrolyzes N-asparagine-linked oligosaccharides from glycoproteins to give free oligosaccharide and a protein containing aspartic acid at the glycosylation site. When uromodulin was incubated with N-glycanase for 72 hours at 37° in 0.2M sodium phosphate buffer, pH 8.6, containing 5 mM EDTA, four N-linked oligosaccharides were released from the glygoprotein. One of these is the active component of uromodulin and inhibits lymphocyte proliferation.

(2) Pronase Digestion - The glycopeptides of uromodulin were isolated by first digesting the protein moiety with pronase for 30 hours at 60°C. The mixture was passed over a Bio-Gel P4 column and 4 fractions which were anthrone positive were obtained. The oligosaccharide with the highest molecular weight had immunosuppressive properties. When this glycopeptide was treated with neuraminidase, the immunosuppressive properteis were lost suggesting that some terminal sialic acid residues are important for the biological activity.

Significance to Biomedical Research and the Program of the Institute: The correlation between the structure of the active oligosaccharide and immunosuppression may help us to understand the role of the immune response in normal and cancer patients. During a program in which we have screened the sera of a large number of patients with various illnesses, we consistently observed that patients with ataxia telangiectasia have high levels of a protein or glycoprotein in their sera which cross-react with a monoclonal antibody prepared against uromodulin. Sera obtained from cancer patients are currently being tested.

Proposed Course of the Project:

We hope to isolate a large quantity of the active oligosaccharide so that we can determine the sequence and the linkage of the sugars. By preparing cyanogen bromide peptides of uromodulin we will be able to determine the nature of the distribution of the active oligosaccharide.

Preliminary studies have indicated that uromodulin inhibits IL-1 (David Rosenstreich, Albert Einstein College of Medicine, personal communication). If this is verified we hope to study the nature of uromodulin IL-1 interaction.

Publications:

None

Project Description

Objectives:

The purpose of this project is to characterize the chemical structure of uromodulin - a glycoprotein isolated from human pregnancy urine - which suppresses the immune response.

Methods Employed:

Affinity chromatography, lectin chromatography, HPLC, gel electrophoresis, chemical modification of the polypeptide moiety, chemical and enzymatic modification of the sugar moiety of uromodulin, ultraviolet absorption spectroscopy, fluorescence spectroscopy, radioautography, automated sugar analysis, sugar sequence and nature of linkage, antigen-antibody interactions.

Major Findings:

Uromodulin is a glycoprotein isolated from human pregnancy urine which has a molecular weight of 85,000 and a minor band of 30,000. Chemical modification with succinic anhydride causes a slight alteration in the tertiary structure of the glycoprotein which affects its antigen-antibody interactions. The lysyl residues are also modified by trinitrophenylation but this modification does not alter the tertiary structure of the glycoprotein.

The apparent maximum in the ultraviolet difference spectrum obtained when the spectrum of uromodulin in 0.1 sodium phosphate buffer, pH 6.5, is compared with the ultraviolet absorption spectrum in 0.1M sodium carbonate buffer, pH 11, occurs at 303nm. In most proteins this maximum occurs at 296nm, which is the maximum of the phenolate ion. This suggests that the tyrosyl residues of uromodulin may be substituted.

Tetranitromethane was introduced into protein chemistry as a reagent which would selectively modify tyrosyl residues. However, TNM may also modify sulfhydryl residues, imidazole residues, indoles and can also induce cross-linking. It is clear that tyrosyl residues of uromodulin are not modified with TNM since there is no absorption band at 430nm which is characteristic of 3-nitrotyrosine. Instead, TNM-modified uromodulin has an absorption maximum at 350nm. The chemical structure of this chromophore has not yet been determined. However, this modified glycoprotein reacts poorly with a monoclonal antibody prepared against native uromodulin.

A third amino acid side chain which was modified in this study was cystine which was first reduced with dithiothreitol in the presence of 6M guanidine hydrochloride. The resulting thiols were reacted with iodoacetate to form carboxymethylcysteine. If the 85K dalton uromodulin consisted of smaller subunits which are held together by disulfide bonds, the reaction conditions employed in this study should have caused dissociation. The opposite in fact occurred. Uromodulin aggregated to form a species which had a very high molecular weight and did not enter the gel. Fluorescence measurements also indicated that reduction-carboxymethylation did not produce unfolding of the tertiary structure.

CONTRACT RESEARCH SUMMARY

Title: Facility for Preparing and Housing Virus Infected Intact and Chimeric Mice

Principal Investigator: Mr. Brian Weatherly
Performing Organization: Bioqual, Inc.
City and State: Rockville, MD

Contract Number: NO1-CB-25005
Starting Date: 9/30/82 Expiration Date: 9/29/85

Goal: Perform a variety of in vivo experiments in mice (up to a colony of 3000 animals) that cannot be performed on NIH campus as designated by the Project Officer. These experiments are to be performed in support of intramural research programs in the Immunology Branch, NCI.

Approach: Experiments are to be performed involving the transfer of normal and neoplastic cells, infection with virus, inoculations of combinations of cells and virus, irradiation with γ-rays or ultraviolet light, preparation of radiation chimeric mice, and thymus transplants. Protocols and details of experiments are to be carried as directed by the Project Officer.

Progress: Performance of this contract has been very satisfactory. A number of experiments involving virus infection, allogeneic lymphocyte transfer, and combinations of the above have been performed in several mouse strain combinations. Thus far, 210 experimental protocols have been performed on the contract. As of April 1, 1984, 8989 mice have been received into the contract facility. 215 ml of cytomegalovirus have been prepared. 2159 radiation chimeric mice have been made, 148 spleen cell preparations have been made, 4554 mice have been injected intravenously, 4461 mice have been injected intraperitoneally, 203 mice have been injected subcataneously, ascities have been recovered from 35 mice, and 786 mice have been tail bled. The mice have been delivered to Immunology Branch laboratories on schedule as requested, and record keeping of stock mice and experimental protocols have been accurate. The Principal Investigator has ordered mice as requested by the Project Officer.

Significance to Cancer Research: This experimental mouse facility is required to support the intramural research programs of the Immunology Branch of NCI in that it provides research that cannot be performed on the NIH campus due to animal restrictions and use of infectious agents in NIH animal colonies. All of the protocols used in the facility relate to a variety of cancer-related issues including studies on radiation chimeras, induction of tumors, passage of tumors, immunological resistance to syngeneic tumor development of models of immune deficiency, and reconstitution of the immune system.

Project Officer: Dr. Gene M. Shearer
Program: Immunology Support
Technical Review Group: Intramural Support Contract Subcommittee A
FY 85 Funds: 0 B

CONTRACT RESEARCH SUMMARY

Title: Maintain an Animal Holding Facility and Provide Attendant Research Services

Principal Investigator: Ms. Leanne DeNenno
Performing Organization: Bioqual, Inc.
City and State: Rockville, MD

Contract Number: NO1-CB-33876
Starting Date: 1/11/83 Expiration Date: 10/31/87

Goal: Maintain colonies of inbred mice (12,000 animals), inbred rats (500 animals), and rabbits (40 animals) and carry out selected breeding protocols with these animals as specified by the project officer. These animals are to be maintained in support of intramural research programs in the Immunology Branch, NCI.

Approach: Colonies of mice, rats, and rabbits are to be housed and fed according to National Research Council standards. Technical manipulations and breeding are to be carried out as directed by the project officer.

Progress: Performance on this contract has been highly satisfactory. The animal colonies have been established and are being maintained according to National Research Council standards. Animal health has, in general, been excellent, and breeding protocols have been satisfactory. Recordkeeping and transferring of animals to and from the NIH Campus have all been satisfactory. Maintenance of frozen products in appropriate freezers has been satisfactory.

Significance to Cancer Research: This animal colony is necessary in support of intramural research programs in the Immunology Branch of NCI. Many of these programs are concerned with the immune response to cancer.

Project Officer: Dr. David H. Sachs
Program: Immunology Support
Technical Review Group: Intramural Support Contract Subcommittee A
FY 85 Funds: $367,302 B

CONTRACT RESEARCH SUMMARY

Title: Characterization of HLA Antigens of Donors' Lymphocytes

Principal Investigator: Dr. Richard Aster
Performing Organization: Blood Center of Southeastern Wisconsin
City and State: Milwaukee, WI

Contract Number N01-CB-33935
Starting Date 7/1/83 Expiration Date: 6/30/88

Goal: To analyze as carefully as possible the cell surface histocompatibility antigens in order to subsequently analyze the relationship between those antigens and the ability of those donors' cells to mount appropriate immune responses.

Approach: Analysis of cell surface antigens is performed by two different detection systems: serology and cellular typing. The serologic analysis is performed using carefully screened alloantisera in assays of complement dependent cytotoxicity. The cellular analysis is done by analyzing secondary restimulation of lymphocyte populations selectively immunized against alloantigens in primary response (PLT), particularly against antigens of the SB locus.

Progress: Serotyping has been performed on 230 cell samples. The quality of the serotyping has been superb. Specifically, the panels of antisera have been continuously updated to include better reagents and new specificities. Of particular importance in the last year has been the careful HLA serotyping of lymphoblastoid B cells and of T cell lines and clones. Such analysis has been crucial for interpretation by NIH investigators of the identity of such lines and (in some cases) the nature of the mutant lines isolated; but such serotyping by the contractor requires expertise and technical skill in performing the assays and interpreting the results.

Twenty four sera have been screened for anti-HLA reactivity.

Progress has been limited in the area of cellular typing, due to departure of the previous principal investigator. Unsatisfactory results were obtained by a subcontracting arrangement with his new laboratory. Recruitment of a new staff member (Dr. D. Eckels) has allowed the resumption of cellular typing effort by the contractor. It is too soon to evaluate the adequacy of performance under this arrangement.

The support of this contract has been essential for many intramural studies including those described in Z01-CB-05067, 05100, 05101, and 05110.

Significance to Cancer Research: Evidence from animal models and from epidemiologic studies in humans suggest that host cellular immune responses are crucial in determining the outcome of neoplastic diseases. Cellular immune responses are under control by genes of the major histocompatibility complex (HLA in man). In order to therapeutically manipulate these cellular immune responses, we must first understand their normal operation and genetic control.

Project officer: Dr. J. Stephen Shaw
Program: Immunology Support
Technical Review Group: Ad Hoc Intramural Technical Review Group
FY 8 Funds: $99,714 A

CONTRACT RESEARCH SUMMARY

Title: Radioimmunoassay and Enzyme Immunoassay of Immunoglobulin Molecules and Antibodies

Principal Investigator: Norman Beaudry
Performing Organization: Hazelton Biotechnologies Corp.
Vienna, Virginia

Contract Number: NO1-CB-44020
Starting Date: 6/30/84 Expiration Date: 6/29/87

Goal: To perform radioimmunoassays of immunoglobulin molecules as well as ELISA assays of antibody molecules in lymphocyte culture supernatants or in biological fluids.

Approach: The contractor is to quantitate human IgG, IgA, IgM, IgE, lambda and kappa light chains, and mouse IgG in various fluids using double antibody radioimmunoassay procedures and reagents defined and supplied by the project officer. Furthermore, the contractor is to measure antibodies produced by lymphocytes stimulated by antigens in vitro. This contract provides critically required research support for the study of immunodeficiencies that are associated with a high incidence of malignant transformation that causes human B- and T-cell leukemias.

Progress: The contractor has established radioimmunoassays for IgG, IgA, IgM, and lambda and kappa light chains of man and IgG of mice. These assays were used to quantitate immunoglobulin and antibody synthesis by human lymphocytes in in vitro cultures. Patients with the adult T-cell-leukemia-associated human T-cell leukemia/lymphoma virus were shown to have a malignant expansion of suppressor T cells that react with a monoclonal antibody anti-Tac that identifies the inducible receptor for T-cell growth factor, whereas patients with the Sezary syndrome have a malignant expansion of helper T cells. The assays for immunoglobulin molecules have been an integral part of studies of a suppressor lymphokine produced by a T-cell line derived using HTLV. The assays of mouse IgG in human plasma were used in studies of the metabolism of anti-Tac in the therapy of adult T-cell leukemia. These studies are defining the nature of disorders of the immune system related to cancer.

Significance to Cancer Research: These studies helped elucidate the abnormalities of the immune system associated with the development of cancer. They have assisted in the categorization of malignancies of the lymphoid system and in defining lymphokines involved in suppression of humoral immunity. They are required for therapeutic protocols involving the use of the anti-Tac monoclonal antibody.

Project Officer: Thomas A. Waldmann, M.D.
Program: Cancer Biology Resource
Technical Review Group: Intramural Support Contract Subcommittee A
FY '85 Funds: $201,782

CONTRACT RESEARCH SUMMARY

Title: Transplantation, Induction, and Preservation of Plasma Cell Tumors in Mice and the Maintenance of Special Strains

Principal Investigator: Martha J. McGowan, Judith Wax
Performing Organization: Litton Bionetics, Inc.
City and State: Bethesda, MD

Contract Number: NO1-CB -25584
Starting Date: 02-22-82 Expiration Date: 1-31-87

Goal: Induction, transplantation, preservation and shipping of plasmacytomas, T- and B-cell lymphomas in mice. Breeding of (congenic) strains of mice, to find genes controlling susceptibility and resistance to the induction of plasma cell tumors by pristane; maintenance of wild mouse colony.

Approach: Maintain a closed conventional colony of inbred and congenic strains of mice, suitable for maintaining mice for long term plasmacytoma induction experiments. Develop BALB/c congenic strains carrying plasmacytomagenesis resistance (PCT-R) genes. Carry out procedures for identifying markers used in the construction of congenic strains. Maintain colonies of pedigreed wild mice. Harvesting and shipment of N_2-frozen transplantable tumors, serum, ascites, tissues, high molecular weight DNA, pedigreed breeders to qualified investigators and collaborators.

Progress: The contractor has continued to produce and develop congenic strains that have been valuable for finding genes that determine susceptibility and resistance to plasmacytoma development. The BALB/cAn·BALB/cJ Raf-$1^{b/b}$ mouse is at N2; F_2 Raf-$1^{b/b}$ in first test are resistant. Contractor is breeding the C X S RI lines from J. Hilgers and completed initial testing that will permit mapping of STS derived resistance genes. Contractor is completing essential experiments to determine the biological effects of indomethacin as an inhibitor of plasmacytomagenesis. Contractor now provides high molecular weight DNA from tumors and mice and, in addition, is converting many of the commonly used lines to tissue culture, which potentially could reduce costs on shipments and *in vivo* transplantation. Contractor continues to perform excellently and deliver tumors, inbred and wild mice, and tumor products to the Laboratory of Genetics and other investigators upon request. The tumor reference bank proved invaluable to us by supplying the tumors for ongoing studies on the *myc*, *abl* and *myb* oncogenes. This colony is probably the only pedigreed source of many of the important representations of the genus *Mus*.

Significance to Cancer Research: Provides essential support for the study of plasmacytomagenesis (carcinogenesis) with the specific goal of determining the genetic basis of susceptibility to tumor induction by mineral oil. Supplies essential biological material for investigators studying the biology of neoplastic plasma cells, tumor immunology, the genetics of immunoglobulins, and immunoglobulin synthesis.

Project Officer: Dr. Michael Potter
Program: Immunology Support
Technical Review Group: Intramural Support Contract Proposal Review Committee
FY 85 Funds: $763,511 B

CONTRACT RESEARCH SUMMARY

Title: Maintenance and Development of Inbred and Congenic Resistant Mouse Strains

Principal Investigator: Ms. Martha McGowan
Co-Principal Investigator: Mr. J. Scott Arn
Performing Organization: Litton Bionetics, Inc.
City and State: Rockville, MD

Contract Number: NO1-CB-25585
Starting Date: 3/1/82 Expiration Date: 1/31/87

Goal: To maintain a colony of inbred pedigreed strains of mice which are needed to support ongoing NCI intramural research in transplantation immunology.

Approach: The contractor maintains a colony of approximately 40 special inbred and congenic resistant strains of mice by pedigreed brother-sister mating. Quality control testing is carried out at each generation by cytotoxicity typing of breeders from each strain. Alloantisera are raised between mouse strains to assist in this quality control typing, and sera and animals are shipped by the contractor to collaborating investigators at NIH and elsewhere.

Progress: The contractor has maintained all inbred and congenic resistant strains of mice in excellent condition. Breeding of each strain and of hybrid strains, recordkeeping, and quality control testing have all been highly satisfactory. A backcrossing program has been instituted for all congenic resistant strains in order to keep the backgrounds of these strains identical. This involves backcrossing of each congenic to the reference background line once every 6-10 generations. This program has also been very satisfactory to date. Twelve new recombinant H-2 haplotypes have been identified during the process of this backcrossing, and these have been bred to homozygosity and established as new valuable inbred congenic strains.

In addition, fusions for hybridoma production are now being done by the contractor, with the purpose of developing monoclonal antibodies to histocompatibility antigens. Antisera for histocompatibility antigen typing have been prepared in various combinations and have been found to be excellent reagents. A series of new strain-restricted typing sera have been produced in order to distinguish each strain in the colony from all other strains. Shipping of animals and sera to collaborating investigators at NIH and elsewhere has been satisfactory. The animals shipped have generally been of excellent health and have provided breeding stock for the production of experimental animals in numerous laboratories. Computerization of records and reports has been performed and has led to efficient data handling. Hybridoma reagents have been produced, stored and shipped starting with cell lines developed by the Project Officer.

Significance to Cancer Research: This animal facility is needed for the breeding and maintenance of these inbred congenic resistant strains of mice. These animals make possible research on individual histocompatibility antigens and, in particular, the role of the major histocompatibility complex in the transplantation of tissues and cells and in the immune response to cancer.

Project Officer: Dr. David H. Sachs
Program: Immunology Support
Technical Review Group: Intramural Support Contract Proposal Review Committee
FY 85 Funds: $552,830 B

CONTRACT RESEARCH SUMMARY

Title: Maintenance of a Feral Mouse Breeding Colony

Principal Investigator: Ms. Martha McGowan

Performing Organization: Litton Bionetics, Inc.
City and State: Rockville, Maryland

Contract Number: NO1-CB-33878
Starting Date: 12-01-82 Expiration Date: 09-30-85

Goal: Induction of mammary tumors with biological (hormones and mouse mammary tumor virus, MMTV) and chemical carcinogens in various feral strains of Mus musculus and other species of Mus. Breeding of (congenic) stains of feral Mus musculus which contain specific geneticaly transmitted MMTV genomes. Maintenance of a pedigreed feral mouse breeding colony.

Approach: Maintain a closed pedigreed colony of feral mice, suitable for long term mammary tumor induction experiments. Genetically introduce specific endogenous MMTV proviral genomes from inbred mouse strains, into the genetic background of the MMTV-germ line negative M. musculus CZII strain. Harvesting and shipment of N_2-frozen primary and transplanted mammary tumors, tissues, and pedigreed feral mice to qualified investigators and collaborators.

Progress: The contractor has continued to maintain all of the colonies of feral mice in excellent condition as well as satisfactorily perform the breeding program, quality control, and maintenance of records. The new common insertion site (designated int-3) for MMTV in the Mus musculus musculus Czech II mammary tumor DNA has been shown by genetic crosses to be located on chromosome 17 near the H-2 locus. Czech II mammary tumors containing an int-3 locus occupied by MMTV express a new species of RNA corresponding to cellular sequences flanking the int-3 locus. Mammary tumors of M. cervicolor popaeus were found to frequently contain insertions of the MC-MTV genome in the int-1 (40%) and int-2 (5%).

The unit II endogenous MMTV genome has been shown to be located on chromosome 6 very near the IgK locus: The gene order was shown by genetic crosses to be IgK/ unit II-c-raf-1-c-k-ras-2. This endogenous MMTV locus was shown to be in 12 different common inbred strains of mice, but absent in the NZB/BinJ inbred strain.

Significance to Cancer Research: Provides essential support for the study of mammary tumorigenesis with the specific goal of dissecting the genetic and molecular interaction between genetically transmitted MMTV genomes and exogenous carcinogens. Provides essential biological material for other investigators studying the biology of mouse mammary tumor virus as well as other classes of genetically transmited retroviral genomes.

Project Officer: Dr. Robert Callahan
Program: Immunology Resource
Technical Review Group: DEA; Ad Hoc Intramural Technical Review Group;

FY 85 Funds: $88,450 B

CONTRACT RESEARCH SUMMARY

Title: Construction and Characterization of Genomic DNA Libraries

Principal Investigator: Dr. J. Norman Hansen

Performing Organization: University of Maryland
City and State: Baltimore, MD

Contract Number: NO1-CB-33934
Starting Date: 08-01-83 Expiration Date: 07-31-86

Goal: Construction of recombinant bacteriophage (libraries) containing entire genomic representation of 5-10 different mouse species and sub-species. Screening of these libraries with a variety of immunoglobulin variable and constant region gene probes for selection of corresponding homologues. Characterization of the isolated clones containing the genes of interest.

Approach: Genomic DNA from species and sub-species selected will be introduced into appropriate bacteriophage following partial digestion with restriction endonuclease enzymes. These libraries will be assayed by filter hybridization to select genes homologous to immunoglobulin genes from inbred stains. Selected genes will then be characterized by restriction enzyme digestion (mapping), hybridization with radioactively labeled DNA probes, and then introduced into plasmid vectors. Preparative amounts of subclones will then be supplied to P.O. for further analysis or sequenced at the contract site as directed.

Progress: To date recombinant bacteriophage DNA libraries have been prepared from five different wild mouse species. All libraries have been screened for genes homologous to immunoglobulin C_K, V_K and V_H sequences and corresponding positive clones have been isolated in each case. A V_H gene family has now been sequenced from three species, and similar studies are in progress on the C_K and V_K genes. One C_K gene has been completely sequenced, and sequencing has been initiated on three others. A number of V_K genes have been isolated from all libraries. These genes are currently being characterized by restriction mapping, and it is anticipated that sequence analysis will commence shortly. The final year of this contract will largely be concerned with further library screening and the detailed sequence analysis of selected genes.

Significance to Cancer Research: One of the possible mechanisms involved in the generation of neoplasia is mutations occurring in structural genes. The present contract supplies materials to permit an assessment of mutational events occurring in multi-gene families in the germline. This analysis will provide information on the occurrence of such diverse events as point mutation, recombination and gene interaction.

Project Officer: Dr. Stuart Rudikoff
Program: Cancer Biology Resource
Technical Review Group: Ad Hoc Intramural Support Contract Review Group
FY 85 Funds: $100,020 B

CONTRACT RESEARCH SUMMARY

Title: Support Services for the Laboratory of Tumor Immunology and Biology

Principal Investigator: Dr. Ronald Gillette

Performing Organization: Meloy Laboratories, Inc.
City and State: Springfield, Virginia

Contract Number: NO1-CP-01018
Starting Date: 6-02-80 Expiration Date: 5-11-85

Goal: To maintain athymic mice bearing human tumor transplants; to prepare purified monoclonal immunoglobulins and fragments and to radiolabel preparations of the aforementioned.

Approach: The contractor houses and maintains athymic mice bearing human tumor transplants for several months; these mice are used in radiolocalization experiments with monoclonal antibodies. The contractor prepares purified immunoglobulins of each of several monoclonal antibodies; immunoglobulins and their fragments are radiolabeled by the contractor. The contractor also maintains tumor and normal human cell lines.

Progress: The contractor has successfully maintained athymic mice, each for several months, bearing human breast, colon, and melanoma tumor transplants. These tumors are monitored for time to tumor appearance, tumor size, and response to monoclonal antibody therapy. The contractor has purified to homogeneity several monoclonal immunoglobulins. Numerous human tissue and normal cell lines have been successfully maintained. The contractor has successfully radiolabeled several monoclonal immunoglobulin and fragment preparations.

Significance to Cancer Research: This contract was necessary for the preparation of purified immunoglobulins to be used in monoclonal antibody research. The radiolabeled immunoglobulins and athymic mouse experiments are necessary preclinical investigations if any of the monoclonal antibodies developed are to be used for localization of tumors in carcinoma patients.

Project Officer: Dr. Jeffrey Schlom
Program: Immunology Resource
Technical Review Group: DEA; Intramural Support Contract Proposal Review Committee

FY 85 Funds: 0

CONTRACT RESEARCH SUMMARY

Title: Hybridoma Assays and Related Laboratory Tests

Principal Investigator: Dr. Ronald Gillette

Performing Organization: Meloy Laboratories, Inc.
City and State: Springfield, Virginia

Contract Number: NO1-CB-33872
Starting Date: 10-01-82 Expiration Date: 09-30-85

Goal: The contractor maintains a hybridoma production laboratory in which selected hybridomas are cloned and screened for specific monoclonal antibody production. Tissue sections are cut from paraffin embedded and fresh surgical specimens supplied by the project officer; these sections are stained using immunoperoxidase techniques.

Progress: The contractor screened hybridoma cell culture supernatants for relevant monoclonal antibody production using solid-phase radioimmunoassays. Mass quantities of tissue culture supernatants and ascites fluids containing useful antibodies were prepared. Quantities of previously existing antibodies were also prepared in this manner. Extracts of human tumor tissue and cell lines were prepared for use in characterizing assays for new and existing antibodies. Additional cell lines were also maintained for use in these assays. Therapy studies using radiolabeled antibodies and fragments in athymic mice bearing human carcinoma xenografts were carried out. Additional antibody fragmentation procedures were also performed. Human tumor tissue specimens were received and embedded or snap frozen for use with additional antibody studies.

Significance to Cancer Research. This contract is needed to process the large quantity of tissues and perform radioimmunoassays needed to screen monoclonal antibodies for specificity. This contract is also needed to produce large quantities of cell culture supernatant fluids and ascites fluids needed for monoclonal antibody research in anticipation of clinical trials and to supply the numerous laboratories requesting these reagents.

Project Officer: Dr. Jeffrey Schlom
Program: Immunology Resource
Technical Review: DEA; Intramural Support Contract Proposal Review Committee

FY 85 Funds: $220,000

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