Division of

Cancer Biology and Diagnosis

1988 Annual Report
Intramural Activities

October 1, 1987
September 30, 1988

U.S. DEPARTMENT
OF HEALTH
AND HUMAN SERVICES

National
Institutes of
Health

National
Cancer
Institute

Bethesda,
Maryland 20892

Division of

Cancer Biology and Diagnosis

1988 Annual Report
Intramural Activities

October 1, 1987
September 30, 1988

U.S. DEPARTMENT
OF HEALTH
AND HUMAN SERVICES

National
Institutes of
Health

National
Cancer
Institute

Bethesda,
Maryland 20892

NATIONAL CANCER INSTITUTE

ANNUAL REPORT

October 1, 1987 through September 30, 1988

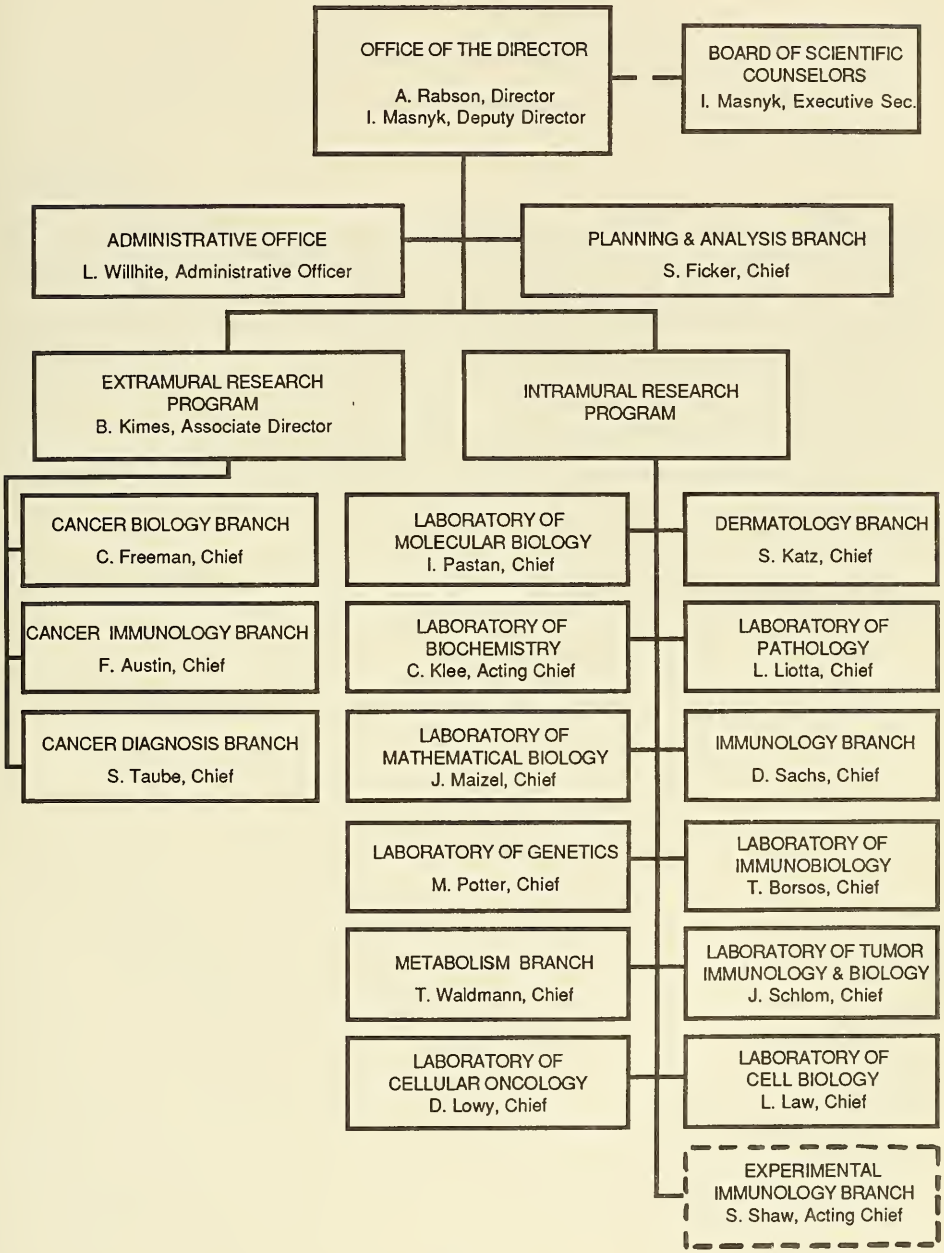
TABLE OF CONTENTS

Project Reports:

Project Reports:

Intramural Research Support Contracts:

DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE



DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE

INTRAMURAL RESEARCH PROGRAM

SUMMARY REPORT OF THE DIRECTOR

October 1, 1987 through September 30, 1988

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. During the past year, an Experimental Immunology Branch was formed. Dr. Alfred Singer was recently appointed Branch Chief. This report will include descriptions of research programs of the intramural laboratories in the division. They are: the Laboratory of Biochemistry, the Laboratory of Cellular Oncology, the Laboratory of Genetics, the Laboratory of Molecular Biology, the Laboratory of Mathematical Biology, the Laboratory of Pathology, the Dermatology Branch, the Metabolism Branch, the Immunology Branch, the Experimental Immunology Branch, the Laboratory of Cell Biology, the Laboratory of Immunobiology, and the Laboratory of Tumor Immunology and Biology.

The research programs in the Laboratory of Genetics, directed by Dr. Michael Potter, focus on the role of genes in neoplastic development, either as targets of mutagenesis (oncogenes) or as genes that modify the host response to tumorigenic agents.

The model system used to study oncogenic activity of transforming retroviruses for hematopoietic tissues, is the pristane conditioned adult BALB/cAn mouse. Injection of Moloney virus (MoMuLV) into pristane conditioned mice induces monoblastic tumors which contain MoMuLV insertions into the c-myb gene. This type of myeloid tumor development can be inhibited by the chronic administration of indomethacin in the drinking water, however, myeloid tumors induced by c-myc containing retrovirus vectors are not inhibited by indomethacin. The mechanism underlying this effect will be investigated further. A major research project has involved the identification and characterization of genes that determine resistance to pristane-conditioned plasmacytoma induction. Three genes have been identified that confer partial resistance: Pctr-1 (distal end, chr4), Pctr-2 (Ly-6 on Chr15), and Pctr-3 (Qa2 chr17). Pctr-1 is the same as or closely linked to Rep-2, a gene whose product appears to determine efficient repair of x-ray induced damage to chromatin.

Others in the laboratory are studying the activation of particular genes in diseased and normal cells in order to understand which genes may play important roles in the development of malignancies, autoimmune diseases and normal differentiation. The immune system has been chosen as the central focus of this research, in particular, the expression of oncogenes, (especially myc, myb, ras, and bcl-2), as well as immunoglobulin and T cell receptor genes. (BALB/c x DBA/2) F_1 hybrids are resistant to pristane induced plasmacytomagenesis, but highly susceptible to pristane-RIM induced plasmacytomagenesis. RIM virus which contains c-myc and ras oncogenes appears to bypass the requirement for the development of chromosomal rearrangements that are essential for myc gene activation. Current experimental data suggests that susceptibility to plasmacytoma induction by pristane RIM virus is determined by a single gene of BALB/cAn origin. Work is underway to isolate this gene. The bcl-2 gene appears to be an important gene in the pathogenesis of follicular lymphomas in man. In the mouse the bcl-2 appears to be expressed in a differentiation stage-specific and cell type-specific manner. Whether excess bcl-2 expression is tumorigenic in mice is being tested using retroviral constructs.

Other investigators have characterized alternative splicing of the c-myb gene in normal and tumor cells in an attempt to determine if alternative transcription of this gene may be important in oncogenesis. A series of experiments has been performed to investigate the biological effects of murine interleukin-6 (IL-6). Initial studies have demonstrated that this factor may be involved in both B- and T-cell development, and may play a role in the generation of primary immune responses to influenza virus hemagglutinin. IL-6 is not active in driving secondary responses.

The relationship of the monocyte/macrophage and B-cell lymphoma lineages to each other in normal hematopoiesis is not clearly understood. The initial approach has been to identify transformed hematopoietic progenitor cells with the potential to differentiate along either the B lymphocyte or macrophage pathways. Two progenitors exhibiting these characteristics and their differentiated progeny have been isolated and characterized. The availability of

these lines offers a unique opportunity to isolate and study genes that: 1) regulate the commitment of cells to a particular lineage, and 2) are involved in lineage-restricted cell functions. The identification of these two categories of genes will be the central focus of future studies.

Investigations on the acute erythroleukemia-inducing spleen focus-forming virus (SFFV) have concentrated on understanding how this virus alters the growth and differentiation of erythroid cells. Erythroid cells from SFFV-infected mice differ from normal erythroid cells in that they can proliferate and differentiate in the apparent absence of the erythroid hormone erythropoietin (EPO) or its receptor. Recent results indicate this is not due to the autocrine induction of Epo or a change in the number of Epo-receptors. Future studies will continue to focus on the mechanism by which SFFV induces erythroleukemia.

Monoclonal antibodies directed against the protein, hen egg white lysozyme c, (HEL) are used as probes to study developmentally regulated antigens in normal and neoplastic development. The X-ray structures of two Fab-HEL complexes for two of these antibodies have been refined. This will allow the study of the molecular mechanisms underlying specificity and affinity in protein-protein interactions.

The Laboratory of Cellular Oncology, directed by Dr. Douglas Lowy, plans and coordinates fundamental research on the cellular and molecular basis of neoplasia. Tissue culture systems and animal models are developed 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 analyze spontaneous tumors from humans and other species for the presence of exogenous genes or altered cellular genes.

Oncogene studies have focused on ras and the epidermal growth factor receptor gene (EGFR). Previous research efforts led to the identification of p21-ras effector region. To identify a cellular ras target, investigators in this laboratory made mutants with a changed phenotype in the effector domain of the p21-ras protein, and mapped the region of p21-ras with which the cellular-protein GAP (GTPase activating protein), interacts to this effector domain. The results suggest that GAP may be a target of p21-ras. Identification of the ras target, and future elucidation of its interaction with the effector region of the p-21 ras protein, should contribute greatly to an understanding of the role of ras in the proliferation of normal and malignant cells. EGF is the cellular honolog of a viral oncogene (erB) product; EGFR is overexpressed in many human tumors. Experiments with a full-length EGF receptor proto-oncogene placed in a retrovirus vector have shown that increased numbers of EGF receptors can contribute to the transformed phenotype. EGF can serve as a competence and progression factor in cells with high numbers of EGF receptors.

In papillomavirus studies, bovine papillomavirus (BPV) E2 protein has been shown to bind to a specific motif present several times in BPV and other papillomaviruses. This motif is an enhancer whose activity depends upon E2. Enhancement requires two or more copies of the motif. Anti-E2 sera have detected three E2 protein products in BPV transformed cells. The smaller forms of E2, which may competitively inhibit enhancement by the full-length E2 product, contain the DNA binding activity but lack enhancer activity. The E7 open reading frame of HPV-16 is the principal viral transforming gene for NIH

3T3 cells. These results support the hypothesis that E7 can contribute to the transformed phenotype in human tumors.

Another ongoing research study is directed towards elucidating the biological functions of the normal human c-fps/fes proto-oncogene and understanding the molecular basis of its oncogenic activity. Recent studies identified a 92 Kda cellular tyrosine kinase (NCP92) as the gene product of human c-fps/fes. Further studies have shown that the transforming activity of c-fps/fes is a direct function of the level of expression of NCP92. The transforming activity of NCP92 could be manipulated at a second level, by modulating its tyrosine kinase activity in vivo. Treatment of infected cells with inhibitor of tyrosine phosphatases significantly increased the transforming activity of NCP92 in a reversible, dose-dependent, and specific manner. In other studies, it was shown that human c-fps/fes is susceptible to oncogenic activation by N-terminal linkage with viral gag sequences, suggesting that perhaps additional mutations are required to bring out the full transforming potential of human c-fps/fes.

Other oncogene studies have involved c-Ki-ras and v-mos. A human embryonal carcinoma cell line can be induced to differentiate into neuron-like cells by various treatments. This cellular differentiation was accompanied by a down-regulation of expression of the amplified c-Ki-ras2 proto-oncogene. This system can be applied to study the epigenetic effect of regulation of oncogenicity.

Studies continued on 3.1kb genomic clone that contains a potential human oncogene originally isolated from an African hepatocellular carcinoma cell line. Using this sequence as probe, four other genomic clones of related DNA sequences have been isolated from hepatomas. Buffalo rat liver cells transformed by these clones were highly tumorigenic in athymic NIH Swiss nu/nu mice. A diverse tumorigenesis was observed; solid tumors (75%) at the site of inoculation and B-cell lymphomas (40%) were induced, with some challenged mice developing both tumors.

Preliminary studies of two new strains of human retrovirus suggest that they may comprise a new type of Human Immunodeficiency Virus (HIV). These viruses exhibited biological characteristics similar to HIV, but their molecular properties differed from HIV-1 and HIV-2. These viruses, as well as prototype HIV-1 and Simian Immunodeficiency Virus (SIV), were also found to replicate and show cytopathic effects on some human B and monocytic cell lines as well as on human T cells. Further characterization is in progress.

The research activities in the Laboratory of Biochemistry, directed by Dr. Claude Klee, focus on elucidating the basic mechanism of cellular regulation. Several groups within the laboratory have directed their efforts towards the study of the regulation of gene expression. One group has continued its analysis of sequence-specific DNA binding proteins in Drosophila with an emphasis on the heat shock proteins and the segmentation gene fushi tarazu. For the heat shock genes, the critical regulatory transcription factor has been purified, and a candidate clone has been isolated and is being sequenced. Concurrently, peptide fragments of the protein are being sequenced in the hope that this will lead to the identification of the authentic gene and enable a detailed analysis of the structure and function of this transcription factor. A biochemical screen of DNA-binding factors that interact with the

Drosophila segmentation gene fushi tarazu has been initiated. One of the factors has been purified to homogeneity, and purification of a second factor has been initiated.

Another research group has devoted its efforts to the study of the regulation of genes associated with the differentiation of muscle. They previously defined the cis-acting elements involved in the regulation of expression of various actin genes. They are now focusing their efforts on the isolation, from nuclear extracts of myoblasts and myotubes, of proteins that bind to these cis-elements. Other studies focus on the regulation of myosin light chain gene expression. Expression of a specific gene in cells induced to differentiate into neuronal cells by nerve growth factor is also under investigation.

The regulation and function of metallothionein gene expression is being studied in the simple unicellular eukaryote, S. cerevisae, and in the more complex cells of mouse and man. Investigators have pinpointed the cis-acting metallothionein regulatory sequences to the level of the individual nucleotides, and have identified several trans-acting regulatory factors. The gene for one of these factors has been cloned from yeast. Additional studies focus on elucidating the role of metallothionein in resistance to the anti-tumor drug cis-platinum.

A structural analysis of the endogenous mouse intracysternal A particle (IAP)-related transposable retroviral elements has been completed, and studies of the regulation of IAP expression at the molecular and biochemical levels are underway. The normal mouse thymus, where levels of IAP expression vary greatly among inbred strains, is being used to analyze the number, identity and genomic environment of active genes. Interaction of the IAP long terminal repeats (LTRs) with nuclear regulatory proteins is also being studied.

Another group in this laboratory is studying the regulation of the synthesis of the extracellular matrix components: collagen and proteoglycan. Earlier studies led them to propose that scurvy-induced fasting leads to hormonal changes that regulate matrix formation. Recent experimental data support this hypothesis, and have suggested that the regulation of growth by vitamin C involves insulin growth factor (IGF-I) and an inhibitor of IGF-I action.

Efforts to develop methods for the early detection of granulocytic leukemia led to the identification of a unique granulocyte maturation factor (GMF) in human sera which appears to be different from granulocyte stimulating factor and transferrin. The purification and characterization of human GMF are underway.

Proteins and their role in the regulation of cellular processes are also topics of interest in this laboratory. One group has been studying the role of calcium in the regulation of stimulus response coupling, with particular emphasis on the mechanism of action of calmodulin. The unique characteristic of this protein is its ability to interact with many different target proteins. Calmodulin interaction with and activation of different targets involves different parts of the calmodulin molecule. Recent studies of the interaction of calmodulin with calcineurin (a calmodulin stimulated protein) have allowed isolation of the calmodulin binding domains. These studies revealed the presence of an inhibitory domain, distinct from the calmodulin binding domain, whose inhibitory effect is prevented by calmodulin binding. Peptide sequence data are being used to isolate cDNA clones of the two subunits of calcineurin

and to synthesize peptide inhibitors of calcineurin which will be used to study the physiological role and mechanism of the calcium regulation of this enzyme.

Another group in the laboratory has concentrated its efforts on the study of the role of myosin phosphorylation in the control of cellular motility in non-muscle cells. Their studies suggest that the stimulation of actomyosin ATPase is not a direct consequence of myosin light chain phosphorylation but is related to the regulation of myosin filament assembly. The identification of this alternative on/off switch will be studied next.

The molecular mechanism of immune reactions, particularly the interaction between MHC (major histocompatibility complex) molecules and the antigen-specific receptors of T cells is another topic of research interest. New methods are being developed to detect the binding of MHC to cell receptors. Synthetic conjugates of anti-MHC antibodies and water soluble polymers are used to present MHC antigens to T cells in a multivalent, unhindered configuration. These "polybodies" may be useful for tumor targeting with MHC antigens.

Mapping of human genes and DNA sequences onto human chromosomes is a topic of increasing interest in the laboratory. A large panel of well characterized human-rodent somatic cell hybrids has been established and Southern analysis of the hybrid cell DNAs with cloned probes is enabling the localization of these sequences to specific human chromosomes. Collaborative efforts are underway to map protooncogenes and genes involved in neurological and autoimmune diseases, transcriptional and cellular regulation, carcinogen and drug metabolism, and neoplastic chromosomal breakpoints. The cloned probes are routinely used to detect the presence of restriction fragment length polymorphism (RFLPs) at these sites, providing a complementary method for mapping by genetic linkage analysis. A third method involves mapping, by genetic linkage studies of kindreds, with nucleic acid probes which detect RFLPs. With these techniques it is now becoming possible to map disease genes for which the underlying defect is unknown and probes are not available. These methods will be used to map disease loci, such as those of the syndrome of thyroid hormone resistance and multiple endocrine neoplasia type I.

Efforts to characterize the highly repeated, interspersed LINE-1 family in the human genome has, for some years, proceeded from the hypothesis that LINE-1s are a family of human transposable elements. This past year, observations in other laboratories demonstrated that LINE-1 elements are transposable and mutagenic in humans, and that transposition causes disease (Hemophilia-A). Recent studies have demonstrated that LINE-1 RNA is a functional mRNA in vitro; that the 5' leader (900 bp) of full length LINE-1s has promoter activity and that a LINE-1 encoded polypeptide is present in human cells. Evidence for expression of one or more LINE-1s is accumulating.

Other groups in the laboratory are involved in the study of the mechanism and control of DNA replication. Studies of P1 plasmid maintenance substantiate a postulated complex role for the product(s) of the plasmid repA gene in both initiating and limiting DNA replication from the plasmid origin. A biochemical analysis of components of the P1 partitioning apparatus has led to the identification of integration host factor (IHF) as a component of that apparatus. IHF serves to increase the affinity of one of two plasmid-encoded partitioning proteins for the P1 centromere. The rare instances of plasmid loss, caused by occasional under-replication or by partition errors, do not

result in viable cured cells. A research project aimed at understanding the molecular mechanism of meiotic recombination in the yeast, S. cerevisiae., has been initiated this year.

Other research efforts have focused on mammalian DNA replication. Earlier success in cloning a cDNA of human β-DNA polymerase has led to the isolation of human genomic DNA spanning the 5'-end of the beta-polymerase gene. Characterization of this DNA defined the transcription start site and the nucleotide sequence of the 100 bp core promoter. Kinetic studies of HIV reverse transcriptase identified early steps in the DNA synthesis reaction mechanism. The enzyme initially binds to the oligonucleotide primer, rather than to the template or template-primer complex. The high affinity of the enzyme for a primer analog may help to develop specific inhibitors of this enzyme.

Research activities in the Laboratory of Mathematical Biology, directed by Dr. Jacob Maizel, encompass studies of macromolecular structure and function, membrane structure and function, immunology and pharmacokinetics. Viral systems are used to model the complex macromolecular processes and organization of normal, infected and transformed cells. Advanced computing techniques are used to study the nucleic acid and protein sequences that embody the information necessary for these systems to function. Graphic representations revealing homology and reverse complementary are coupled with numerical methods to aid the prediction of secondary structure, splicing, promoters, and recombination in nucleic acid molecules. Roles for genes and proteins are deduced by comparison with databases of sequences of known proteins. Many of these studies are only possible through the use of the computing factilities at the Advanced Scientific Computing Laboratory, Frederick Cancer Research Facility.

Sequences of picornaviruses have been examined for relationships within the family and to other known and hypothetical proteins. A single base change at position 472 in Sabin polio type 3 correlates with reversion to neurovirulence; it also changes the predicted stem and loop secondary structure. 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.

Investigators in this laboratory have developed an algorithm to identify potential T cell sites based on helical amphipathicity. Peptides predicted by this method are being used in collaborative studies to design T cell vaccines against malaria and against the AIDS virus. An examination of DNA mapping strategies has been initiated in order to identify strategies best designed to map chromosomal size DNA. The overall goal of the mapping is to generate a library of clones of DNA fragments that covers the entire DNA and to obtain partial but sufficient information about the fragments in order to determine their order in the original DNA.

Studies on the primary structure of beta 1-4 galactosyltransferase (1-4 galtransferase) are continuing. The results of these studies show that the amino-terminal 72 residue peptide is missing from the secreted and soluble form of the enzyme. The remaining 320 residue protein (the soluble form) carries the catalytic domain. The topology of the Golgi and cell 1-4 galtransferase is

predicted to be similar to a class of membrane proteins, like the transferrine receptor, which have an NH_2 terminal "stem" structure extending from the luminal side of the transmembrane bilayer to the cytoplasmic side. The bulk of the protein structure, which carries the main function, faces the lumen side. Studies on the structure-function relationship of galactosyltranserases and their modifiers, like alpha-lactalbumin have been initiated. The cDNA sequences of 1-4-galtransferase and alpha-lactalbumin, are engineered in such a way that the expression of the cDNA has the potential to produce either soluble and secreted forms or membrane anchored forms of the protein.

A new approach has been taken to the protein folding problem. The procedure is to generate all possible compact conformations and to assess their quality in a general way, based upon what has been observed in globular protein crystal structures. This effectively leads to "fuzzy" protein conformations, with only the major features of the residues that are close to one another. In this way it should be feasible to consider the folded conformations of both small proteins and RNA of unknown structure.

Molecular modeling has been proceeding in four area: membrane channels, small peptides, DNA double helix, and DNA-protein interactions. Experimental data is combined with computer calculations to yield better conformational prediction methods. For example, conformational models have been developed for the channel-forming membrane proteins: melittin, magainin, pardaxin, δ lysin, and PGLa; these have assisted in understanding how they lyse cells and form channels. Comprehensive experiments in which repressor-operator binding is perturbed by changing the base sequence with every possible single base pair substitution and a number of amino acid substitutions, provide information about the strengths of the individual atomic DNA-protein interactions, and how to best place the repressor in its most favorable orientation within the major groove of the operator region. In an attempt to understand DNA conformations in control regions, the strength of strand pairing in the promoter region has been studied. Stability of the DNA double helix was determined in the vicinity of the promoter. For a large set of prokaryotic promoters, the region just before mRNA start sites is significantly less stable. There appears to be a strong correlation between the free energy change and the mutation type in the promoter region.

GELLAB is a computer based system for the analysis of sets of 2D electrophoretic gels. GELLAB incorporates sophisticated subsystems for image acquisition and processing, data base manipulation and graphics as well as statistical analysis. The objective of creating an exportable version of GELLAB is being actively pursued. The new system, GELLAB-II, will run under UNIX and use X-windows for portable interactive graphics, and in the future will be exported to VMS and CRAY systems.

The feasibility of delivering monoclonal antibodies via lymphatic vessels for diagnosis and possible treatment of lymph node metastases and lymphoma has been demonstrated in this laboratory. The initial studies were done in animals, and clinical trials have been initated to assess the effectiveness of monoclonal antibodies in patients with T-cell lymphoma, small cell carcinoma, non-small cell carcinoma, and breast cancer. The goal is to develop a sensitive, non-surgical diagnostic method.

Three algorithms (HAL, HALP, and HALCO) have been developed with which to analyze secondary structural characteristics of peptides and to assess their potential for antigenicity or for other functional characteristics. The results have been applied to HIV envelope protein. Liposomes containing antiviral drugs have been conjugated to monoclonal antibodies targeted to HIV-infected cells. These "targeted" liposomes are being tested in culture and in animals for their ability to react with HIV-infected cells. The goal is to deliver anti-viral agents directly to the infected cells.

Studies of membrane structure and function focus on the mode of action of the G protein of Vesticular Stomatitis Virus (VSV), the HN and F proteins of Sendai Virus, and the HA protein of influenza virus. The development and application of label-fracture cytochemistry has continued. Label-fracture is a molecular cytochemical approach to map, at a resolution better than 10 nanometers, the position of molecules on the surfaces of biological membranes. This method has been applied to a variety of cells, receptors, antigens and lectin binding sites. Over the past year a new method-Replica-Staining-Label-Fracture (or fracture-flip) was developed. The fracture-flip method is based on the splitting of bimembranes by freeze fracture and on the stablization of the exoplasmic halves of split membranes by carbon evaporation. The work on localization of CD3 and CD4 antigens in human lymphocytes was continued using fracture-flip. In other studies label-fracture was used to locate a glycoprotein related to the multi drug resistance of certain cell lines.

Development continued on the computer system (SAAM) for the simulation, analysis, and modeling of biokinetic systems. Both batch (SAAM) and interactive (CONSAM) versions were ported to the UNIX-PC workstation, making the capabilities of this software available on a computer costing less than $5,000. A new graphics capability was added to the CONSAM programs; and the theoretical development of parameter identifiability and estimability continued. The SAAM project also carried out collaborative research efforts, involving a large number of national and international investigators, in the analysis of data in many diverse fields.

The Laboratory of Molecular Biology, directed by Dr. Ira Pastan, uses genetic and molecular biological approaches to study gene activity and cell behavior and to develop new approaches to the diagnosis and treatment of human disease.

Resistance to multiple drugs is a major impediment to the successful chemotherapy of human cancers. A model system was developed to investigate the genetic and biochemical basis for this multidrug resistance (MDR) phenotype. Studies using this system showed that resistance results from expression of the MDR1 gene which encodes a 170,000 dalton membrane glycoprotein (P-glycoprotein) which is a multidrug transport protein. Expression of MDR1 RNA and P-glycoprotein occurs in normal kidney, liver, colon, and adrenal and in tumors derived from these tissues which are intrinsically resistant to chemotherapy as well as several other tumors. Acquired drug-resistance in childhood leukemia, neuroblastoma, rhabdomyosarcoma and in pheochromocytoma may be associated with increased MDR1 RNA levels. New strategies are being devised to overcome resistance due to expression of the MDR1 gene.

The production of transgenic mice constitutes a powerful new tool to study expression of multidrug residence gene. The MDR1 gene has been microinjected into one-cell mouse embryos and germ line transgenic mice established.

Expression of the MDR1 gene can be detected in the normally drug-sensitive bone marrow. Studies are underway to determine if transgenic mice overexpressing the MDR1 gene in bone marrow are more resistant to chemotherapy.

The epidermal growth factor receptor (EGFR) is overexpressed in many human tumors implicating it in the pathogenesis of some cancers. To test the role of the EGFR in cancer, a retrovirus encoding the EGFR was constructed and shown to cause EGF dependent transformation of 3T3 cells. Current studies focus on elucidating the mechanism of regulation of the EGFR gene. These studies should should lead to new strategies of cancer treatment. To further study the role that the EGF receptor plays in normal and transformed cells, transgenic mice have been generated that carry germ line-integrated EGF receptor genes. These mice are being analyzed for EGF receptor gene expression, and scrutinized for the appearance of cancer.

A major project in this Laboratory is the continued development of immunotoxins and oncotoxins for the therapy of cancer. The approach has been to attach Pseudomonas exotoxin (PE) or genetically modified forms of PE to monoclonal antibodies (MAbs) or growth factors to create cell-specific cytotoxic agents. Native PE attached to mAb OVB3 is being evaluated for its efficacy in treating women with ovarian cancer. Transforming growth factor (TGF) reacts specifically with cells bearing EGF receptors. Because overexpression of the EGFR is observed in many cancers, mutant PE molecules have been fused with TGFα, and the resulting chimeric protein, (TGFα-PE40) produced in E. coli. TGFα-PE40 is very cytotoxic to cells with EGF receptors. Similarly, because the IL2 receptor is overexpressed in adult T cell leukemias (ATL), a chimeric protein, IL2-PE40 was created. IL2-PE40 is cytotoxic to leukemic ATL cells, and is being evaluated in clinical study in patients with ATL.

Studies of the regulation of growth in mammalian cells have continued. The approach has been to investigate growth inhibitory activity as detected by DNA-mediated gene transfer. Two techniques, magnetic affinity cell sorting (MACS) and fluorescence activated cell sorting (FACS) have been used in conjunction with gene transfer to measure transient growth regulation mediated by exogenous DNA sequences. Of particular interest has been development of an efficient transfection protocol for human embryo fibroblasts in which cDNA libraries are screened for genes involved in senescence and tumor suppression. Evidence has been obtained for growth regulation by RNA polymerase III-encoded transcripts. At the DNA level, cytotoxicity has been found to follow introduction of retroviral, e.g., HIV, long terminal repeat sequences. This finding may have implications for mechanisms of viral pathogenicity.

Fibronectin is a major cell surface and extracellular matrix glycoprotein involved in cell adhesion and migration. A synthetic peptide, containing the key fibronectin adhesive recognition sequence has been developed that can inhibit tumor cell metastasis in an experimental animal model. Since renal clearance of the peptide was very rapid, new peptide conjugates are being developed as a means to increase its time in the circulation. The molecular basis of fibronectin function is being analyzed in an attempt to develop improved inhibitors of fibronection-mediated adhesion and migration.

The biosynthesis, regulation, and function of receptors for cell adhesion proteins are also being analyzed. Membrane binding molecules for fibronection and collagen have been characterized. The beta subunit of both mouse and human

fibronectin was synthesized as a smaller precursor that required a lengthy maturation period. Maturation resulted in an activation of fibronection-binding function and secretion onto the cell surface. Experimental data showed that levels of both alpha and beta subunits of the human fibronectin receptor were regulated by transforming growth factor β. Roles for fibronectin receptor were shown in embryonic amphibian gastrulation and human platelet function. A collagen-binding protein, found to be a heat-shock protein, decreased after malignant transformation of activated cells. This protein (hsp 47) has been characterized and partially sequenced. Two putative biosynthetic precursors of this hsp47 protein were identified, both of which were fully biologically active. This collagen-binding protein shows the diversity of heat-shock protein function and of the mechanisms of receptor functional activation.

Cultured mouse fibroblasts, malignantly transformed or treated with TPA or growth factors, synthesize and secrete the 39,000 Mr precursor to cathepsin L in large amounts. This purified procathepsin L contains mannose 6-phosphate, the lysosomal recognition marker. It is processed intracellularly to give two specific lower molecular weight forms with a lysosomal localization. Secreted procathepsin L can bind to the mannose 6-phosphate receptor of many cells and be endocytosed and processed intracellularly. Procathepsin L uptake reduces the efficiency of antigen presentation in antigen-presenting cells, thereby interfering with immune response. A functional procathepsin L mouse gene was cloned and the promoter identified. A human procathepsin L cDNA has also been cloned and sequenced.

The Chinese hamster ovary (CHO) fibroblast is being used to study the genetics and biochemistry of some aspects of the behavior of cultured cells. This work has emphasized morphology and its relationship to growth control, and the manner in which cyclic AMP regulates cell growth an gene expression.

Neoplastic transformation produces many changes in cell physiology, some of which have been studied using morphologic techniques. Technical improvements have streamlined the morphologic hybridoma screening processing. For example, P170, the product of the human MDR1 gene, was localized using antibody MRK16 in the GI tract, adrenal, kidney, and liver, and in capillaries in the central nervous system. The reactivity of monoclonal antibody C219 that reacts with an epitope of P170 present on the inner surface of the plasma membrane was studied. The antibody cross-reacts with other proteins, such as the heavy chain of cardiac and slow-twitch muscle myosin, in human, monkey, and rat tissues.

Studies of mechanisms of the transport of thyroid hormones into animal cells have continued to yield information on the characterization and regulation of expression of a thyroid binding protein, p58. A full-length p55 cDNA was inserted into a Harvey murine sarcoma virus-derived vector (pHTBr) and transfected into NIH 3T3 cells. Nine stable cell lines expressing p55 were identified which provide a tool to study further the function of p55 and its role in T_3 action. A cellular hormone binding protein (p58) was purified to homogeneity. Two monoclonal antibodies to p58 were isolated and are being used to investigate its role in the intracellular transport of T_3. Recent studies have shown that c-erbA protein is the T_3 nuclear receptor. Antipeptide antibodies to the human placenta c-erbA (hc-erbA-β) protein were prepared and are being used to study the tissue distribution and subcellular location of the T_3 nuclear receptor.

Genetic regulatory mechanisms are being studied in E. coli. The mechanism by which the Gal repressor protein negatively regulates the expression of the gal operon of E. coli. continue to be an area of interest. It has been proposed that the two repressor molecules bound to the spatially separated operators associate, giving rise to a higher order structure containing a DNA loop with bound repressor, CRP and DNA polymerase. The DNA in this complex is altered and inactive for transcription. Recent experimental data are consistent with this model.

Additional studies focus on the mechanism by which cyclic AMP binds to its receptor protein CRP to induce a conformational change in the protein, and how the cyclic AMP-CRP complex modulates gene transcription. Cyclic AMP-CRP complex, and not free CRP, binds at or near many promoters of E. coli in a sequence-specific way and either represses or activates transcription. Several classes of mutations in the crp gene were studied which identify the amino acids participating in the cyclic AMP-induced conformational change in the protein. Using a DNA bending vector developed in this laboratory, it was found that CRP binding induces DNA bending at all test promoters with a CRP binding site whether the promoters are activable or repressible by CRP. Additional data strongly support the idea that CRP activates RNA polymerase by a direct protein-protein contact and not by sending a signal through DNA.

The role that protein degradation plays in control of cell growth continues to be studied using mutants defective in ATP-dependent and independent protein turnover. E. coli lon mutants are defective in cell division regulation after DNA damage, and overproduce capsular polysaccharide. It was shown that an unstable positive regulator of capsule synthesis, RcsA, is stabilized in lon mutants. RcsC, a negative regulator of capsule synthesis, is a membrane protein and thus may act as a sensor for environmental signal controlling capsule synthesis. The gene (termed clpA) for the ATP-binding subunit of a new two component ATP-dependent protease was isolated. While cells devoid of clpA are healthy, the Clp protease seems to act as a back-up to lon in degrading abnormal proteins. A third protease function has been identified, and the gene responsible has been mapped to a location on the E. coli chromosome.

To study properties of origin specific initiation of replication in vitro, an enzyme system was developed to study the interaction of RepA and DNaA proteins with the P1 origin DNA. Recent data have shown that the RepA protein binds to plasmid control locus incA and simultaneously to the repA promoter in the origin region, causing the intervening DNA to loop. DNA looping very likely could provide the mechanism by which RepA bound to incA might exert repression.

The Laboratory of Pathology, directed by Dr. Lance Liotta, is responsible for all the diagnostic services in anatomic pathology, neuropathology, cytopathology, electron microscopy, and hematopathology. In addition, active research programs in various areas of experimental pathology are carried out.

Approximately 6,000 surgical specimens or biopsies (approximately 60,000 slides) were accessioned in the past year. Approximately 1,000 specimens of fresh human tissues, including the eyes, were furnished to NIH scientists in various laboratories. A diagnostic immunohistology service is provided for clinical diagnosis and research. Clinicopathological studies in pediatric neoplasms, soft tissue sarcomas, acquired immune deficiency syndrome (AIDS), mast cell disease, human breast cancer diagnosis, and lung pathology are in

progress. Investigators in the Pulmonary and Postmortem Section are conducting a detailed review of interstitial fibrotic lung disease. A comprehensive study of the pulmonary pathology of the Acquired Immune Deficiency (AIDS) is currently being conducted based on surgical and autopsy lung pathology material from AIDS patients seen at the NIH.

The Cytopathology Section provides diagnostic services in cytology; during the year 7,500 cytology specimens were accessioned. In addition, the staff collaborates in various clinicopathologic research projects. The Ultrastructural Pathology Section provides diagnostic electron microscopy services for the Clinical Center; approximately 200 cases were accessioned; over 150 were processed and diagnosed.

Studies in this laboratory in collaboration with the Pediatric Oncology Branch led to the identification of a unique, previously uncharacterized tumor of childhood, peripheral neuroepithelioma, demonstrating conclusively that it is distinct from classic childhood neuroblastoma. The ability to distinguish this tumor from other, histologically related tumors, has enabled the improvements in the design of clinical treatment protocols. n-myc oncogene expression is an important factor in neuroblastoma prognosis and biological behavior. n-myc expression has been reported sporadically in other childhood tumors. However, recent studies have demonstrated that n-myc expression is not associated with any other histologically related childhood tumors as was previously thought. Thus, detection of n-myc expression is a reliable diagnostic marker of neuroblastoma. Similar studies have resulted in an improved prognostic classification of childhood rhabdomyosarcoma. The ability to correlate oncogenes with prognosis has important diagnostic and treatment implications.

The Biochemical Pathology Section carries out research on immunochemistry of complex carbohydrates, with emphasis on the determination of carbohydrate structures of glycoproteins and analysis of oligosaccharide mixtures by gas chromatography/mass spectrometry (GC/MS). 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. Related studies include the development of antibodies against oligosaccharide haptens; immunochemical studies of cell surface glycoproteins and sequencing of antibodies of the group-A streptococcal carbohydrate V_H genes.

This laboratory has made several important advances in understanding cell invasion and metastasis, and unraveling the biochemical and molecular genetic mechanism of tumors. Research studies have identified three biochemical factors enhanced in actively invading tumor cells: a) a new type of matrix receptor (laminin receptor), b) a new type of collagenase which cleaves type IV collagen of the extracellular matrix, and c) a new type of autocrine motility factor (AMF). The laminin receptor is a cell surface protein to which laminin (a major component of the basement membrane) binds specifically. Clinical studies have consistently demonstrated a higher content of exposed laminin receptors in breast cancer cases with two or more lymph nodes positive for metastases. Monoclonal antibodies to the laminin receptor have been coupled to adriamycin containing liposomes. Recent data demonstrated the effectiveness of those "targeted" liposomes in killing human breast cancer cell. Clinical trials are being planned.

Investigators have isolated and characterized molecular clones of laminin receptor and of several different collagens. During the past year, they have extended the sequence analysis of the laminin receptor gene, obtaining more amino terminal sequences. They discovered that there is more than one laminin receptor gene which may encode more than one protein expressed in the cell. Related studies have shown that murine cells also have multiple laminin receptor genes. Studies of the regulation of the laminin receptor gene indicate that differentiated cells express less laminin receptor mRNA than do related, undifferentiated precursor cells. This is consistent with the general observation that aggressive undifferentiated tumors which metastasize express more laminin receptor.

Type IV collagenase is an important basement membrane degrading metalloproteinase that malignant cells produce and secrete to facilitate their invasion into surrounding tissue. Antibodies against type IV collagenase react with actively invading breast cancer cells; and this has important diagnostic significance. Type IV collagenase has been purified and subjected to amino acid sequencing. Antibodies against type IV collagenase have been employed in the development of an ELISA assay for human serum samples. A complete cDNA clone encoding human type IV collagenase has been isolated. Significant progress has been made in the understanding of the mechanism of activation and the substrate cleavage site.

Type IV collagenase is markedly augmented following ras oncogene transfection. A model system using ras transformed rat embryo cells was used to study the correlation of basement membrane collagenolysis with metastatic propensity. Rat fibroblasts transformed by ras^H are highly metastatic and produce high levels of type IV collagenase; while cotransfection of ras^H plus the adenovirus type 2 EIA gene yields cells which are highly tumorigenic, but non metastatic and fail to produce type IV collagenase. Data from these studies support a genetic linkage of type IV collagenase expression with the metastatic phenotype in this rodent system.

In previous studies, investigators in this laboratory identified and purified autocrine motility factor (AMF) that is produced by actively invading tumor cells. This material, produced and responded to by metastatic tumor cells, appears to exert its action by perturbing membrane phospholipid metabolism of the cell, acting through "G" proteins. A cDNA clone for human AMF is being characterized. The measurement of AMF in urine samples appears to be an accurate marker of transitional cell carcinoma of the bladder.

A variety of molecular genetic techniques are being employed to identify specific genetic elements whose expression is altered in metastatic cells. One gene, NM23, has been found to be associated with the metastatic process. It is expressed by two low metastatic melanoma cells lines at levels at least 10-fold higher than in five related, highly metastatic cell lines. The association of high levels of NM23 expression with low metastatic potential has been confirmed in additional experimental systems both in vitro and in vivo. Thus, metastasis may not result simply from the acquisition of invasive traits, but may involve the loss of certain cell functions. The level of expression of the NM23 product showed a highly significant inverse correlation with human breast carcinoma aggressiveness (as measured by numbers of axillary lymph nodes positive for metastases). This data suggest that NM23 may be a putative suppressor gene.

Experimental studies in the Hematopathology Section continue on the immunological characterization of malignant lymphomas. All Clinical Center 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, to distinguish new clinicopathologic entities, and as a basis for immunotherapy. A study of the pathologic features of HTLV-I associated lymphomas has continued. Prospective studies of all lymphomas in geographic regions with differing incidences of adult T cell leukemia/lymphomas are undertaken to discern factors which make an impact on the incidence of HTLV-I and HTLV-I associated disease.

Lymphocytes activated with IL2 both in in vivo and in vitro are being used for the experimental treatment of human malignancies. The mechanism of action of this therapy is being investigated to determine whether those lymphokine-activated killer cells (LAK) infused actually migrate and infiltrate into tumors, or whether other effector cells mediate the observed tumor regression. It is hoped that information from this study can be used to develop a reliable method of predicting clinical response.

Molecular genetic techniques are being exploited to improve the diagnosis of human lymphoproliferative disease. It is now possible to confirm the T- and B-cell lineage of diffuse, aggressive lymphomas by detection of appropriately rearranged T-cell receptor or immunoglobulin genes. Similarly, other types of malignant lymphoma can be identified by these techniques. A t(14;18) chromosomal translocation, involving each of the known DNA breakpoint clusters of the bcl-2 gene, has been associated with follicular lymphomas. Four DNA breakpoint clusters were identified and the location of the breakpoint may influence the course of the disease. Recent evidence suggests the existence of an additional breakpoint. Attempts are underway to clone the DNA sequences at new breakpoint regions and link them to the bcl-2 transcriptional unit.

The recently discovered gene encoding the delta chain of the human T cell receptor lies within an actively rearranging region of the T cell receptor alpha locus. Using DNA probes of this region, a T-delta gene rearrangement has been identified as a frequent, early event in precursor T cell neoplasms (adult lymphocytic leukemia). In the course of this study, two new productive V-delta genes were discovered which together with the previously identified V gene appear to comprise the entire delta V repertoire.

It is now known that frequent relapse of patients with follicular lymphoma is a consequence of clonal expansion of daughter cells derived from a common stem cell. Thus, despite the "clinical" remission achieved by therapy in most patients, residual lymphoma cells must persist. Molecular genetic techniques have enabled the development of a new assay to detect occult lymphoma which should aid in the early detection of disease recurrence.

Molecular genetic technqiues are being employed to study the regulation of cell growth by transferrin receptors and oncogenes. 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 (TFR) expression becomes constitutive. A model system has been developed which will enable researchers to study the ability of iron to regulate TfR mRNA levels of the message. In related studies it was shown that

HTLV-I infected T cells express 10-20 fold more TfR on their surface, but that this is due to a redistribution of the receptor from cytoplasm to surface and not to an increased receptor synthesis. Further, the TfR in these cells is poorly internalized, poorly phosphorylated and cannot deliver iron to these cells.

Antisense oligomers have been used to study the role(s) of specific c-myc nuclear proteins in cell activation and proliferation. Induction of IL-2 receptors, transferrin receptors, DNA polymerase α and blastogenesis in mitogen-treated T cells do not require c-myc protein. Using an in vitro DNA polymerase α assay system, it was determined that both c-myc protein and a nuclear protein termed K_i-67 are required for DNA polymerase α activity.

A novel method for identifying sequence-specific DNA binding proteins was developed and continues to yield exciting information. A particular strain of the gibbon ape leukemia virus is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with a complex of at least two cellular proteins. One of these components interacts strongly with DNA but without full specificity. The second component confers additional specificity, probably by allowing additional protein contact.

The Metabolism Branch is directed by Dr. Thomas Waldmann. A broad range of clinical and laboratory investigations are carried out in an attempt to define and characterize factors involved in the control of the human immune response, with emphasis on the molecular analysis of the abnormal cells of T and B cell leukemias, and the development of molecular techniques to facilitate somatic gene therapy. Other studies focus on determining the physiological and biochemical effects that a tumor produces on the metabolism of the host. Special emphasis is placed on insulin-like growth factors that play a role in the hormonal control of normal and malignant growth, and on the regulatory role on intermediary metabolism played by proline and its metabolite pyrroline-5-carboxylate.

A major thrust has been the molecular analysis of the rearrangements of immunoglobulin genes and T cell receptor genes involved in the control of the synthesis of immunoglobulin and T cell receptor. A similar analysis has revealed that the abnormal lymphocytes of patients with T-gamma lymphoproliferative disease represent a clonal proliferation of T lymphocytes. Current studies have focused in molecular analysis of transforming genes that translocate into the immunoglobulin gene locus in certain B cell neoplasias, and T cell receptor locus in certain T cell tumors. The t(14;18) (q32;q21) chromosomal translocation associated with follicular lymphoma has been studied and the candidate transforming gene was identified. Recent studies revealed that the t(14;18) translocation results in the deregulated expression of the chimeric transforming gene/immunoglobulin gene (bcl-2/IgH) mRNA transcripts with somatic mutations in the bcl-2 protein coding region.

Molecular analysis of a T cell chronic lymphocytic leukemia has revealed a translocation between chromosome 14 q11, and the allelic chromosome 14 at band q32. This represents a translocation that joins a J region gene of the T cell receptor α locus with a putative cellular transforming gene at 14q32 that may contribute to the malignant transformation.

The molecular analysis of transacting factors that mediate lymphoid specific gene transcription has continued. The scientific focus of this work is the purification of the transactivating factors, the cloning of the genes encoding these factors and the definition of their mode of action at the molecular level. The promotors of all Ig variable genes and Ig heavy chain enhancers contain a critical octamer DNA motif which, together with a TATA motif, is necessary and sufficient for lymphoid-specific promotor activity. Additional studies have demonstrated the presence of a ubiquitous octamer binding protein (NF-A1) and a lymphoid-specific octamer binding protein (NF-A2), presumed to mediate the lymphoid-specific activity of Ig promoters. The putative human NF-A2 structural gene (termed oct-2) has been cloned. The oct-2 gene sequence revealed a 60 amino acid domain that is homologous to the "homeo box" domain of proteins which are important for cell type determination and development in yeast and Drosophila. New evidence demonstrated that this domain of the oct-2 protein is required for DNA binding; this represents the first demonstration that a mammalian homeo box gene encodes a sequence-specific DNA binding protein. Recent evidence indicates that the octamer motif may control the expression of other lymphoid-specific genes besides Ig, notably the bcl-2 gene involved in the pathogenesis of human follicular lymphomas. Using the cloned oct-2 gene, it should be possible to determine the range of genes which are expressed exclusively in lymphocytes under the transcriptional control of the octamer motif.

A primary goal of one research group in the Branch has been to determine the rules that govern which structures on a protein molecule will be likely to be recognized by T lymphocytes. Such rules would be valuable in understanding the processes of immune T cell recognition as well as in identifying the appropriate components of artificial vaccines. These studies have identified several factors that appear to be important in determining immunodominance. Besides interaction with major histocompatibility (MHC) molecules, the mode by which the antigen is processed into fragments for T cell recognition is also important. The data suggest that the products of natural processing of the protein are larger than was previously postulated and contain structures which hinder binding to certain MHC molecules or to the T cell receptor. In addition, the intrinsic structure of the antigenic site is important; amphipathic helices have a higher than random chance of being immunodominant. A computer program has been developed to locate such structures in protein amino acid sequences, and this methodology has been applied to two practical cases of vaccine development. In one case, this approach was used successfully to predict the major site that T cells recognize in one of the proteins of the malaria parasite, and a protein fragment derived from this sequence has been shown to be capable of stimulating an immune response to malaria in an animal model. Similarly, two T cell recognition sites were identified on the AIDS virus envelope, and immunization with these elicits enhanced antibody responses to the whole envelope when injected into monkeys. These sites were also recognized by human T cell from volunteers who had been immunized with a recombinant vaccinia virus expressing the HIV envelope. Other immunodominant sites in the envelope protein have been identified, including a very large one within which different MHC types of mice each recognize different overlapping regions. In other studies, a recombinant vaccinia virus and transfectants expressing the HIV envelope gene were used to induce specific cytotoxic lymphocytes (CTL) against the HIV envelope. Using synthetic peptides, it has been possible to identify the first known CTL recognition site in the AIDS virus. However, such sites are very limited; of five different class I MHC

molecules that could have presented HIV envelope sites, only one appeared to be used.

The development of techniques to facilitate gene therapy in man has continued to receive major emphasis. Investigators have been successful in transferring the gene for the enzyme adenosine deaminase (ADA) into ADA deficient T cell lines and have totally corrected the biochemical defect in these cells in vitro. Using autologous bone marrow transplantation in lethally irradiated monkeys, it has been possible to transfer the gene for human ADA into these monkeys. Although expression of the enzyme could be detected, the level and duration of expression were insufficient to attempt human application. A new approach using cultured lymphocytes and fibroblasts as cellular vehicles for gene transfer is being explored. With T cells as the carriers of transduced genes, mice continue to express human ADA, at high levels for many weeks. Studies with lymphocytes as gene therapy targets will continue to receive major emphasis.

Activation of resting T cells induces the synthesis of interleukin-2 (IL-2) as well as high affinity IL-2 receptors that are not expressed on resting T cells. There are two classes of IL-2 receptors, a very high affinity and a low affinity receptor. Previous studies in this Branch identified two peptides that bind IL-2, the 55-kDa peptide, (Tac protein), and a novel (p75) peptide. A multichain model has been proposed for the high affinity IL-2 receptor in which both the p55 Tac and the p75 IL-2 binding peptides are associated in a receptor complex. Essentially all T cell functions require T cell activation and expression of both the p55 Tac and the p75 peptide. The precursors of lymphokine activated killer cells and natural killer cells, however, express only the p75; activation of these cells requires only the interaction of IL-2 with the p75 but not the p55 peptide. Over the past year, evidence has developed to suggest that a third 95-110 kDa peptide is associated with the Tac peptide in the high affinity receptor.

The Tac peptide is expressed by a number of types of malignant mononuclear cells, by T cells involved in certain autoimmune disorders, and by T cells participating in allograft rejection and in the graft vs host reaction. Since the Tac peptide is expressed on the leukemic cells in patients with adult T cell leukemia (ATL), a monoclonal antibody against Tac (anti-Tac) has been used to treat patients with this disease. Initial results were promising, and plans are underway to increase the number of patients receiving this therapy. In preliminary clinical studies, there was virtuallly a complete elimination of early renal allograft rejection in patients who received anti-Tac in addition to conventional immunosuppressive therapy. Other studies have shown that when certain radionuclides are chemically linked to the anti-Tac molecule its efficiency is increased even further: Anti-Tac has been coupled to a bacterial toxin (Pseudomonas exotoxin) and the resulting immunotoxin, while successful in an animal model system, proved to be hepatotoxic in humans. Recombinant DNA techniques were used to modify the PE gene to eliminate this toxicity. In addition to its use in ATL, anti-Tac has been shown to be effective in treating other neoplasms not responsive to traditional therapy, certain autoimmune diseases, and in prolonging the survival of organ transplants. Recombinant DNA technology has been used to produce "humanized" anti-Tac molecules which retain their anti-tumor activity without the potentially undesirable side effects often associated with reagents produced in mouse cell lines.

An enzyme-linked immunosorbent assay has been developed for the measurement of the soluble forms of Tac peptide in the serum. Elevated levels of soluble Tac protein were found in the sera of patients with ATL, Tropical Spastic paraparesis, AIDS, hairy cell leukemia, and in patients with certain autoimmune diseases. Elevations of Tac protein in patients with ATL and hairy cell leukemia were indicative of tumor burden; favorable response to therapy was associated with reductions in the serum level of Tac. Elevations of Tac protein in serum were also associated with allograft rejection episodes in patients with liver and heart-lung transplants.

Research has progressed from the purification, immunologic characterization, sequencing, and cloning of uromodulin, an 85 KD immunosuppressive glycoprotein derived from pregnancy urine, to now focusing on the characterization of glycosylation patterns responsible for the biologic activity of uromodulin. A panel of related oligosaccharides has also been purified and characterized. These compounds block T cell responsiveness in vitro, induce a delayed inflammatory response in vitro, induce PGE2 synthesis in vivo, and compete with binding to IL-1, IL-2, and tumor necrosis factor (TNF). Recent studies have shown that IL-2 and TNF directly bind to class II antigens and regulate their expression in vitro; and that this binding is mediated by oligosaccharides expressed by class II molecules. Further studies will explore the immunoregulatory activity of these structurally unique compounds.

The insulin-like growth factors (IGF-I and IGF-II) play an important role in the normal growth process in vivo. Molecular cloning of the human type II IGF receptor revealed an 80% sequence homology with the bovine mannose 6-phosphate (man-6-P) receptor suggesting identity of the new receptors. Biochemical studies carried out in this laboratory support this conclusion, and that the binding sites on the IGF II and Man-6-P are distinct. Additional experiments were undertaken to define interactions of two classes of ligands (IGFs and lysosomal enzymes) for binding to the IGF-II/Man-6-P receptor. A panel of lysosomal enzymes inhibited the binding of ^{125}I-IGF-II to the purified receptor. This inhibition appeared to result from a change in the binding affinity for IGF-II. This suggests that IGF-II can modulate the targeting of lysosomal enzymes to lysosomes. Additional studies in rats revealed that the receptor is present in multiple tissues and is developmentally regulated, being high in fetal tissue and declining postnatally to much lower levels by day 20.

The regulatory function of proline and its metabolite pyrroline-5-carboxylate continue to be explored. It has now been established that pyrroline-5-carboxylate (P5C) is a nutrition-dependent intercellular communicator which modulates the effects of certain growth factors in transmembrane signaling. Initially it was demonstrated that P5C is a constituent of human plasma and that levels of P5C peak following meals. Thus, the physiologic variation in P5C concentrations can transmit nutrient-dependent information to peripheral tissues. P5C entry into cells is mediated by a group translocation mechanism which transfers oxidizing potential across cell membranes. P5C reductase plays a role in this translocation. Of special interest, P5C acts synergistically with platelet-derived growth factor in stimulating phosphoribosyl pyrophosphate formation. These studies show that P5C serves as a novel mechanism for integrating nutritional influences with growth factor-stimulated cellular events to produce physiologic or pathophysiologic responses.

The Dermatology Branch, directed by Dr. Stephen Katz, 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 Branch also serves as Dermatology Consultant to all other services of the Clinical Center (approximately 1500 patients are seen in consultation each).

A major area of study continues to be on the role of the epidermis as an immunological organ. Epidermal Langerhans cells are derived from precursor cells in the bone marrow and play an essential role in many of the immunological reactions affecting the skin. An epidermal Interleukin 1-like cytokine has been identified which may serve as a second signal in the generation of T cell responses as well as a proinflammatory agent affecting other cells - especially neutrophils. When epidermal Langerhans cells are cultured for 2-3 days they become very potent antigen presenting cells. When these cultured cells are modified with hapten, they can generate primary immune responses. The sensitized T cells thus generated respond preferentially to the same hapten in vitro. Research efforts have focused on the characterization of murine dendritic thy 1 positive epidermal cells. The data conclusively support the finding that these cells are of T cell lineage and may represent an extra thymic source of T cells in nude and as well as normal mice. The other major focus of this laboratory has been the study of the function of class II MHC bearing keratinocytes which appear in humans and mice during cell-mediated reactions in the skin. Recent studies demonstrate that these class II bearing keratinocytes induce specific immunological unresponsiveness.

Laboratory studies of autoantibody-mediated skin diseases have continued. The antibodies in these diseases define molecules in the normal epidermis. Thus, these studies contribute to our understanding of the structure and function of the normal epidermis as well as to the pathogenesis of these diseases. Antibodies from all patients with the autoimmune disease pemphigus foliaceus (PF) bind a protein found in desmosomes, and define a calcium-sensitive epitope on a desmosomal protein complex. Pemphigus vulgaris antibodies (PV) bind a complex that is different from the PF antigen complex, however, recent data demonstrate that they share certain biochemical similarities. A cDNA clone that synthesizes part of the bullous pemphigoid (BP) antigen has been isolated and used to show that the mRNA encoding BP antigen is about 9kb. The peptide sequence of the C terminal end of the molecule has also been deduced.

Another research effort has focused on the study of the human dermal microvascular endothelial cell, its cultivation, characterization and the modulation of is phenotypic and functional characteristics. Human dermal microvascular endothelial cells (HDMEC) can be induced to undergo extremely rapid differentiation, and form elongated tubes that contain lumens. Recent studies have shown that the active substance inducing this angiogenesis, is laminin. The immunologic phenotype of HDMEC, has been defined. These cells possess the cell adhesion molecules ICAM-1, LFA-3 and CD44. ICAM-1 can be upregulated in IL-1 and TNF; LFA-3 and CD44 are not. In addition, these cells also express CD13, CD9, CD6 (previously thought to be present only on T cells). The adhesion of T cells to HDMEC is due to expression of cell adhesion molecules on both types of cells.

Investigators in this laboratory 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. The efficacy of isotretinoin as a chemopreventive agent is being studied further in a series of seven patients with xeroderma pigmentosum and nevoid basal cell carcinoma syndrome. Preliminary results are favorable and long-term low-dose isotretinoin therapy is now being used for cancer chemoprevention in these patients.

Studies of the role of DNA repair processed in cancer, aging and neurodegenerative disorders continue. 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 primary neuronal, muscular, and retinal degenerations are also being studied. Assays have been developed to assess the biological effectiveness of DNA repair in cells from patients with the Cockayne syndrome (CS) and xeroderma pigmentosum (XP).

The Laboratory of Tumor Immunology and Biology, directed by Dr. Jeffrey Schlom, carries out a broad range of laboratory investigations that include 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 human mammary, colon, and lung carcinomas.

Progress is being made in the generation and characterization of monoclonal antibodies (MAbs) that are reactive with human tumor cells, and clinical trials have been initiated using radio-labeled MAb 72.3 to detect and localize carcinoma lesions in breast, colorectal, ovarian, and lung cancer patients. Therapeutic trials using higher doses of radioactive B72.3 are also being conducted. B72.3 is also being used to identify breast cancer cells in cytologic preparations of human effusions and in fine needle aspirations using a new immunohistochemical technique. Some tumor masses contain tumor cells that do not express detectable levels of tumor associated antigens. This expression can be enhanced by the addition of interferon. Clinical trials using recombinant interferon to enhance MAb uptake in breast cancer patients are being planned. One of the problems observed in studies using radiolabeled MAbs for detection of human tumors is the relatively low percent of the injected dose of monoclonal antibody per gram of tumor. Recent studies in animal model systems for melanoma and hepatoma, as well as clinical studies involving hepatoma patients have suggested that there is enhanced deposition of radiolabeled antibody in tumors pretreated with external irradiation. Studies have been initiated to adapt this observation to the development of improved methods of MAb binding to tumors. One approach to the treatment of patients with peritoneal carcinoma may be direct infusion into the peritoneal cavity of radiolabeled antibody conjugates. This method may improve the percent of the injected MAb dose per gram of tumor, and may lead to less radiolabeled antibody in the bone marrow and, therefore, less toxicity to the patient. A project is underway to develop an *in vivo* model system to study immunotherapy of human carcinoma in the peritoneal cavity.

Basic studies of the development and differentiation of normal and malignant breast tissue are carried out in animal and human systems, with particular emphasis on the role of hormones and growth factors. One research project is designed to evaluate the nature of lactogenic hormone receptors and the factors

(including other hormones) that affect the binding of the hormone and its receptor. The prolactin receptor has been purified and partially characterized. The nature of the interaction of Tamoxifen with membrane-bound receptors related to the lactogen receptor is being studied.

Studies have continued on the role of derangements in tropomyosin (TM) expression and utilization in neoplastic transformation. Neoplastic transformation secondary to expression of retroviral oncogenes is correlated with the production of TM-deficient, structurally defective, microfilaments in both fibroblasts and epithelial cells. The derangement results either from suppression of synthesis and utilization of TM (fibroblasts), or from overexpression of actin (epithelial cells). Consistent abnormalities in tropomyosin expression were observed in human breast cancer cell lines, suggesting that derangement of TM expression may be a frequent event in human mammary neoplasia.

The expression and phosphorylation of the phosphoprotein, prosolin, in human peripheral lymphocytes (PBL) is being investigated. Its expression was found to be correlated with the S-phase of the cell cycle. The availability of prosolin only during S-phase may provide a regulatory mechanism by which naturally occurring cytolines may terminate lymphocyte growth, through activation of antiproliferative processes linked to phosphorylation of prosolin, without inhibiting activation of G_0 or G_1 cells.

Another research group in the laboratory is interested in the biochemistry of oncogenes. An essential part of their work concerns the identification and occurrence of transforming growth factors (TGF), studies on their mechanism of action, and their functional relationships to transforming oncogenes. Elevated levels of alpha TGF and its message can be detected in 65% of primary human breast tumors, and the growth of human mammary tumor line MCF-7 can be inhibited following treatment with either anti-TGF or anti-EGF receptor antibodies. The mechanism of transformation of cells by oncogenes has also been approached by isolating cells which are resistant to transformation by specific oncogenes. Revertants that are resistant to transformation by *ras* and certain other oncogenes have been studied with respect to monovalent cation transport. These studies have demonstrated a transformation-related alteration in ion transport in a variety of cells transformed by *ras*, including human and murine epithelial and fibroblastic cells. New methods useful for the generation and isolation of revertant cells resistant to transformation to other oncogenes are being developed.

Efforts to identify and characterize the genetic changes associated with the development of mammary tumors are underway. Studies have focused on the isolation and characterization of recombinant cDNA clones for the integration site (designated *int*-3) for murine mammary tumor virus (MMTV) in virus-induced mouse mammary tumors. MMTV integration at this locus activates the expression of a 6.6- and 2.4-kb species of RNA. Other studies have shown that there is a significant difference in the frequency with which MMTV integrates at *int*-1 in BALB/cfC3H compared with C3H mammary tumors. Similarly, there is a significant difference in the frequency of MMTV (RIII) integration event at *int*-2 in BALB/cfRIII compared with RIII mammary tumors. These results suggest that the inbreeding program for high tumor incidence in C3H and RIII mouse strains has selected for host genetic mutations that affect the frequency of *int* gene activation. Several mammary hyperplastic outgrowth lines (HOGs) have been

developed which should enable investigators to begin to dissect at a genetic level the mutational events resulting in the development of preneoplastic and malignant mammary epithelial phenotypes.

Efforts to identify and characterize frequent genetic changes associated with primary human breast tumor DNAs have continued. The int-2 gene is related to the fibroblast growth factor gene family. Amplification of the int-2 gene has highly significant association with patients who subsequently develop a local recurrence or distal metastases. However, in contrast to a published report from a group outside the NIH, the investigators in this laboratory did not find any significant association between amplification of the c-erB-2 gene and the prognosis in breast cancer patients.

The Cellular Biochemistry Section studies the mechanism of cell growth and differentiation by cyclic AMP (cAMP). The physiologic role of cAMP in the growth control of a spectrum of human cancer lines, including leukemic lines, and v-rasH oncogene-transformed NIH/3T3 cells is demonstrated by the use of site-selective cAMP analogs. These cAMP analogs, which can select either of the two known cAMP binding sites of the cAMP receptor protein, induce potent growth inhibition, with no toxic effect, in a spectrum of cancer cell lines at micromolar concentrations. This suggests that the site-selective cAMP analogs, substituting for endogenous cAMP and binding to protein kinase, suppress the cancer cell growth not via directly inhibiting cell division by rather by promoting cell differentiation.

Others in this section have postulated that deregulation of oncogene expression may be a possible general mechanism for the induction of neoplasia in humans. Their efforts have concentrated on the cellular homologue of the ras gene. In a study of more than 200 human primary breast carcinomas, an elevated expression of c-rasH in correlated with the progression and poor prognosis of the disease. Interestingly, an amplified or rearranged c-rasH gene has not been detected in human mammary carcinoma. The goal of this study is to elucidate the mechanism of oncogene involvement in the neoplastic transformation and progression.

Investigators in the Cell Cycle Regulation Section focus on the role of growth regulation factors in the development of neoplasia. To this end they have studied the production of growth modulators by normal and neoplastic mammary epithelium from rodent and human tissues and fluids. Four such activities, two stimulatory and two inhibitory factors have been identified and are being characterized. Mammary derived growth factor (MDGFI) appears to be a unique growth regulatory factor whose overproduction favors rapid breast cancer cell growth. A second growth promoting factor, transforming growth factor alpha (TGFα), is produced by normal cells and in some breast tumors. Its production in rodents appears to be regulated by estrogenic hormones, and may be correlated with the presence of estrogen and progesterone receptors.

Research activities in the Laboratory of Cell Biology, directed by Dr. Lloyd Law, focus on the identification and characterization of tumor-specific transplantation antigens (TSTA). Three TSTA have been purified from the chemically induced Meth A sarcomas. One of these antigens is a major cytosolic heat shock protein (hsp) consisting of two isoforms (84 and 86 kDA); the other is a less abundant cytosolic, but highly immunogenic, 82 kDa protein. All of the Meth A TSTAs appear to have counterparts in nontransformed cells which are

indistinguishable by molecular mass, isoelectric point or by the individual anti-TSTA sera from the corresponding Meth A TSTA. The genes coding for both the 84 and 86 kDa hsp-related TSTAs have been isolated, and recent evidence suggests that the active gene is located on chromosome 17. The chromosomal location of hsp 84 relative to the MHC class I region is currently being mapped. Full length cDNA has also been sequenced for the 86 kDa hsp from Meth A tumors, and the DNA sequences related to this protein have been assigned to chromosomes 12, 11, and 3. The gene coding for hsp 86 from normal mouse cells is currently being isolated. Previous studies showed that the 82 kDa and the hsp related TSTAs are present in the cytoplasm and at the cell surface of in vitro cell cultures. Current data suggests that the presence of these TSTAs at the cell surface is due to cell lysis followed by binding of these relatively abundant proteins to the cell surface.

Another group in this laboratory has focused its efforts on the role of the host immune response to the growth and metastatic spread of malignant melanoma. Their results have demonstrated that various murine melanomas share a common cell surface antigen which can elicit tumor rejection. This antigen, B700, a glycoprotein of 65 kDa m.w., is related biochemically and immunologically to a normal melanocyte protein, perhaps a differentiation antigen. The immune response elicited by B700 is mediated through cytotoxic antibody production and not through a cellular response. Other studies focus on determining the role of cell surface urokinase in the development of the metastatic spread of tumors. The molecular mechanisms involved in the regulation of mammalian pigmentation and its activation by environmental stimuli are also being explored. Melanin production appears to be regulated primarily through a post-translational activation of latent enzyme and that one or more genes normally expressed by melanocytes are repressed in transformed melanocytes, while other melanoma-specific genes are activated following transformation.

Another aspect of the work in this laboratory is a study of the highly conserved class I regulatory element (CRE), which is involved in the regulation of class I genes. Three independent nuclear factors have been identified that bind to overlapping, but distinct regions of the CRE. Interferon (IFN) induces transcription of class I genes through the use of an interferon consensus sequence (ICS), and the binding of nuclear factors to this region is being studied. Results indicate that the ICS binds a constitutive nuclear factor present in various cells irrespective of IFN treatment. This sequence is shared by a number of IFN inducible genes and may represent a basic structure needed for IFN mediated transcriptional induction.

Other investigators have continued to analyze T cell hybridomas from H-2^d mice that recognize the hen egg white lysozyme (HEL) sequence 107-116 in association with the I-E^d molecule, as a mode for studying T cell antigen recognition. HEL binding studies have been carried out with I-E^d and I-E^d purified protein. The results indicate that a series of peptides containing region 105-120 binds I-E^d and not I-A^d, and that the shortest peptide (residues 107-116) binds to I-E^d about 10-fold better than the other peptides studied. The binding of a peptide to the Ia molecule appears to be a prerequisite in determining its immunogenicity. An analysis of the correlation between the interaction of various synthetic peptides encompassing the entire HEL sequence with I-Aa^k molecules and the T cell response to these peptides has revealed three immunodominant T cell epitopes. Two were recognized in association with I-A^k molecules and another with I-E^k molecules.

During the last year, investigators in the Laboratory of Immunobiology, directed by Dr. T. Tibor Borsos, have made major advances in several areas of research. Studies in this laboratory have contributed significantly to our understanding of the activation of complement. Their efforts have focused on investigating the subcomponents of C1, the first component of the classical complement cascade. C1 is composed of three subcomponents: C1q (the recognition component), which activates C1r (the internal activating proenzyme), which in turn activates C1s (the proenzyme for activating the C cascade). Data from other groups suggested that C1 activation was spontaneous, autocatalytic, controlled by an inhibitor, and that antibodies only accelerated this process. However, results in this laboratory yielded contradictory evidence for C1 and C1s activation. Studies during the past year focused on the mechanism of activation of C1r, and yielded similar results. Activation of C1r, as with C1s is concentration dependent; the rate enhanced by the addition of already activated C1r, and is inhibited by serine protease inhibitors. These results were almost identical with those obtained with C1s and suggested that they were inconsistent with a spontaneous intramolecular autoactivation model, and instead suggested intermolecular activation by contaminating serine proteases or by activated C1r. Recent studies of C1q structure/function confirm an earlier hypothesis; that the deviation of native C1 dose-response curve from linearity is due to concentration dependent dissociation of C1q into smaller fragments, only a fraction of which was associated with C1r and C1s in activable form.

Other research efforts during the past year have been the characterization of an attractant for human neutrophils that is produced by LPS-stimulated human blood mononuclear cells. This attractant is called monocyte-derived neutrophil chemotactic factor (MDNCF). MDNCF has been purified and is being characterized. Of great interest is the observation that MDNCF did not attract human monocytes. The amino acid composition and N-terminal sequence of MDNCF has been determined. MDNCF has sequence similarity to several other peptides that are released in response to tissue injury. Of particular interest is the similarity to PF-4 and CTAP-III, two peptides released from platelets in response to aggregation or stimulation. An ELISA assay has been developed to detect MDNCF in biological fluids. Preliminary flow cytometry data show that fluoresceinated MDNCF binds to human neutrophils, but not to lymphocytes, with only a very low level binding to monocytes; consistent with the unresponsiveness of monocytes to MDNCF.

A relatively new area of research in the laboratory has been the study of human tumor suppressor genes. Last year's annual report described their discovery that sporadic human renal cell carcinomas and small cell lung carcinomas were characterized by a loss of DNA sequences on the short arm of chromosome 3 (3p). Genes that lead to cancer when the function of both copies has been eliminated by mutation have been referred to as tumor suppressor genes or recessive oncogenes. Analysis of additional samples of renal cell carcinoma and small cell lung carcinoma has confirmed the loss of DNA sequences on chromosome 3p. Studies of familial renal cell carcinoma have confirmed the concept that a gene that predisposes to renal cell carcinoma is located on chromosome 3p. Immunological techniques have been used to improve the method for detection of deletions in human solid tumors. A strategy for isolation of the renal cell carcinoma gene was developed and 3000 clones isolated from a chromosome 3 library. Recently, studies designed to detect chromosomal loss in human adrenal cortical tumors have revealed a loss of DNA on chromosomes 11 and 17.

These results suggest that there may be a gene(s) located on chromosome 11p and/or on chromosome 17 that plays a role in the development of adrenocortical carcinoma.

The Immunology Branch, directed by Dr. David Sachs, carries out studies of the major histocompatibility complex (MHC) and its role in transplantation in animal models and in man.

A breeding program is carried out for the maintenance of pedigreed inbred and congenic resistant strains of mice for the development of new recombinant H-2 haplotypes for structural and functional studies of genes within the MHC. New strains bearing recombinant MHC haplotypes are utilized for the production of new reagents and probes to study the fine structure of the MHC. One recent recombinant, the C3H.KBR, derived during the backcrossing of the C3H.SW to C3H, produced a surprisingly high anti-Qa reactivity when immunized against C3H.SW. Future studies may provide a clue to the natural function of ligand interactions with the Qa molecule. Another new recombinant, the A.TH-Q1b, has made possible for the first time, an analysis of the A.TH anti-A.TL reactivity in the absence of a Qa-1 difference.

The induction of intentional mixed chimerism across MHC barriers has been investigated as a means of producing specific transplant tolerance. Reconstitution of irradiated animals with a mixture containing T cell depleted syngeneic marrow plus non-T-cell depleted allogeneic marrow has been found to produce complete allogeneic chimerism while avoiding GVHD. This model may be useful in the treatment of leukemia, since the procedure does not appear to eliminate the graft-vs-leukemia (GVL) effect. The use of monoclonal anti-T-cell subset antibodies substitute for part of the irradiation procedure has been explored in an attempt to reduce radiation induced toxicity.

Partially inbred strains of miniature swine have been developed as a large animal pre-clinical model for transplantation studies. Five new recombinant haplotypes have been identified within these herds; three of these have been bred to homozygosity. By serologic, biochemical and genetic data, all of these recombinants appear to have split class I from class II loci. Studies using the new recombinant MHC haplotypes have demonstrated the importance of class II antigen matching in determining the outcome of vascular allografts such as kidney transplants.

A molecular approach to the analysis of class II genes in miniature swine has been initiated. Southern blot analyses indicate that genes corresponding to each of the major human class II loci are present in the pig genome, suggesting that conservation of this allele may be due to its utility in response to a common pathogen. Class II genes from the pig herds have been isolated and are being characterized and used in in vitro and in vivo transfection studies. Without exogenous immunosuppression, miniature swine matched for class II and transplanted across a class I plus minor antigen difference frequently develop specific tolerance. The mechanism of this tolerance may involve a specific depletion of class I reactive helper T-cell populations. A new radiation regimen with fractionated irradiation permits successful transplantation of MHC mismatched bone marrow. However, non-T-cell depleted bone marrow grafts across full MHC barriers were found to produce aggressive lethal GVHD. The effect of mixing autologous plus allogeneic marrow in the reconstituting inoculum is

being examined. The result may have applicability to transplantation in humans.

The Experimental Immunology Branch, directed by Dr. Alfred Singer, carries out laboratory investigations in lymphocyte differentiation and regulation, the cell biology of immune responses; transplantation biology; the structure, regulation, and function of genes involved in the immune response; and tumor immunology. A flow cytometry laboratory is maintained which supports over 60 research projects for investigators in the EIB and elsewhere at the NIH. During the past year, instrument modifications and procedures have been implemented to enable three-color immunofluorescence analyses. In addition, a project to construct a micro-vax based list mode data analysis work station has been initiated.

Lymphocyte differentiation and regulation is being studied by several different approaches. A series of murine models have been developed to study lupus-like autoimmunity. These models are characterized by autoantibody production and activation of suppressor T cells that are selective in their effect on $CD4^+$ T helper cells, mimicking the situation observed in humans with systemic lupus erythematosus. These studies may provide a better understanding of immune regulation and dysregulation in autoimmune disease.

Studies of T lymphocyte responses in HIV^+, asymptomatic individuals indicate that approximately 50% of these patients exhibit selective $CD4^+$ T helper cell functional defects without any AIDS symptoms and without loss of $CD4^+$ cell number. A defect of antigen-presenting cell (APC) function was not observed in these individuals. Many patients in various stages of disease exhibited a selective loss of $CD4^+$ T helper function but retained $CD4^-$ T helper function to HLA alloantigens. These studies provide evidence for an alternate ($CD4^-$) T helper pathway that may be utilized for developing immunotherapy strategies in HIV^+ patients).

The role of the T cell receptor (TCR) $\alpha\beta$ heterodimer in antigen recognition has been analyzed. The nature of polymorphism and allelism were re-evaluated in the minor lymphocyte stimulating (Mls) system. Mls determinants are defined by the ability to stimulate primary proliferative T cell responses between major histocompatibility complex (MHC) identical cells. In contrast to what had been previously postulated, recent evidence suggests that the Mls system is composed of the products of at least two unlinked loci, with no evidence for structural polymorphism at either locus at the present time. Further analysis of T cell receptor (TCR) expression in Mls^c reactive T cells demonstrated a striking association of this reactivity with expression of the Vβ3 gene product. The role of endogenous lymphokines in T cell activation has also been evaluated. Experimental evidence demonstrates that interleukin 4 (IL4) acts as an autocrine growth factor for type 2 T-helper cells. Further studies support the conclusion that T cell activation mediated through the T cell receptor complex is dependent on IL4. Endogenously produced IL4 may normally down regulate its own production at the level of gene transcription or message stabilization.

Characterization of B lymphocyte subpopulations and membrane molecules continues. A previously undescribed mouse alloantigen (Ly-39.1) has been identified which is expressed on B lymphoblasts. Its expression is controlled

by a gene located centromeric to H-2 on chromosome 17. This antibody may allow efficient separation of resting and activated B lymphocytes.

Heteroantibodies with dual specificity can be constructed by chemically crosslinking an antibody against the cytotoxic cell receptor (CCR) to an antibody against a tumor cell antigen. By linking the target cell directly to the CCR, the heterocrosslinked antibodies promote target cell lysis. Current studies focus on methods of identifying and generating subsets of cells from human peripheral blood which can be targeted against pathogenic cells for potential therapeutic purposes. For example, human peripheral blood lymphocytes, targeted with heterocrosslinked antibodies against ovarian cancer, have been shown to be effective in killing ovarian cancer cells both in culture and in animal studies. Plans are underway to adapt this technique to the human situation.

Characterization of two major subsets of human T lymphocytes; naive and memory T cells, has continued. This work demonstrates clearly that these subsets differ in major ways both in phenotype, (in expression of at least 6 different cell surface molecules including three involved in adhesion) and in function (only the memory cells respond to recall antigen). Furthermore, the memory cells produce much more gamma interferon in response to a variety of stimulatory agents than do naive cells. Studies continue to be directed at elucidating the mechanisms of T cell recognition, with particular emphasis on antigen-independent T cell adhesion as a critical early step in the process of recognition. Major progress has been made in understanding the functional role of the ICAM-1 molecule which appears to function as a ligant for LFA-1. ICAM-1 has been purified; two binding sites have been implicated in its adhesion function.

Confrontation with thymic Ia determinants is necessary for the development of class II specific $CD4^+$ T cells, but is not for class II-specific $CD8^+$ T cells. Thus, the differentiation requirements of phenotypically distinct T cell subsets are not identical. Characterization of the differentiation and recognition specificities of $CD8^+$ class I specific T helper cells has continued. These T cells differentiate intrathymically and are tolerized by thymic MHC determinants, but display a remarkably narrow response repertoire that so far includes only class I MHC allogantigens and viruses.

The roles performed by CD4 and CD8 molecules in T cell function have also been examined. CD8 performs a signalling function in the activation of $CD8^+$ T cells that requires multivalent cross-linking of the molecule CD8. Recent evidence suggests that CD4 and CD8 differ in their response to activation of protein kinase C; contradicting the concept that they are functionally equivalent molecules.

Several research projects study the cell biology of the immune response. Both specific and non specific T cell derived signals play a role in the activation of B cell antibody responses. Two distinct soluble T cell derived proteins appear to be involved in this activity: Interleukin 4, and an antigen-specific and MHC-restricted soluble factor. This latter factor was purified. Current experimental data suggest that these factors are secreted from cytoplasmic granules in activated helper T cells to target B cells.

Antigen-presenting cells coated with several different antibody heteroconjugates present antigen to T cells at concentrations which would normally be too low to elicit a response. With splenic B cells, heteroconjugates containing anti-Ig, anti-class I and anti-class II, but not anti-$Fc\gamma R$ enhances antigen presentation. Antigen targeted to surface immunoglobulin was endocytosed and presented more rapidly than antigen targeted to MHC molecules. Preliminary studies have shown that heteroconjugates can aid in the development of antibody responses in vivo. Heteroconjugates containing anti-class I or anti-class II, but not anti-IgD antibodies were able to enhance antibody production, suggesting that the enhancement was not occurring via B cells.

Studies on the mechanism of lymphocyte-mediated cytotoxicity have shown that red blood cells are killed by both cloned cytotoxic T lymphocytes and helper T cells when linked to these cells by their T cell receptor. The mechanism of this cytotoxicity involves the release of a potent lytic agent, cytolysin, from the secretory granules of cytotoxic lymphocytes. Cytolysin appears to bind to target cells prior to the lytic event. The subsequent lysis of the stable intermediate is calcium dependent. Studies on tumor target cells show that cytolysin can cause a sublethal membrane damage which can be repaired by the cells. The role of secretory granule proteases in mediating target cell DNA breakdown during killing by cytotoxic T lymphocytes has been examined further. The results suggest a role for serine proteases in target cell DNA breakdown.

A model system has been developed to explore the possibility that lymphocyte granule exocytosis occurs into a synapse-like zone created between the lymphocyte and an attached triggering cell. When allowed to settle onto glass surfaces coated with anti-T3 antibody, cloned mouse CTL and T helper cells exclude the penetration of antibodies from a region of substrate underneath themselves. The formation of these synapse-like junctions is not blocked by drugs which block energy production or interfere with cytoskeletal protein function, and thus may be formed by diffusion of membrane antigen.

In other recent studies, it was shown that IL-4 specifically induces a marked loss of binding of complexes to B lymphocyte $Fc\gamma R$ II. Their data suggest that IL-4 regulates the binding of complexes to B lymphocyte $Fc\gamma R$ by inducing some form of alteration of the receptor. This effect could play a role in the regulation of B lymphocyte responses by preventing $Fc\gamma R$ II mediated down-regulation.

Studies of the role of immune cell function in transplantation biology have continued, in particular, the effects of cyclosporin A (CsA) on lymphocyte function in allograft retention have been studied. In vivo studies in mice indicate that lower doses of CsA selectively abrogate $CD4^+$ T helper function and activate T suppressor cells, whereas higher doses of CsA abolish both $CD4^+$ and CD^+ T cell functions and activate non-T suppressor cells. Preliminary studies in humans treated with different doses of CsA suggest similar patterns of T cell inactivation. These findings may be useful in determining optimal doses of CsA for treatment of transplant patients. Other experiments indicate that murine cytomegalovirus (MCMV) infection can synergize with a graft-versus-host (GVH) reaction to make the GVH more severe. Current studies are underway to elucidate the mechanism of the synergistic effects of CMV infection and GVH disease in human bone marrow transplantation.

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL), may play a significant role in mediating allogeneic marrow graft rejection. It was found that: a clone murine CTL population was sufficient to reject an allogeneic marrow graft and that the rejection was specific for MHC gene products expressed by the donor marrow corresponding to the cytotoxic specificity of the clone. In a subsequent study it was shown that administration of anti-CD3 to mice results in T cell activation which produces extramedullary hematopoiesis in the spleen, and enhances marrow engraftment. These results may have direct application to the human situation.

Studies in murine marrow chimeras have suggested that MHC mismatched marrow transplantation is associated with immunoincompetence of the T cell arm of the immune response. To evaluate whether T cell maturation processes are comparable in mouse and man, the development of T cell responses in the rhesus monkey has been studied. The preliminary results suggest that the generation of T cells in marrow grafted primates may not be the same as in the mouse. Such differences would potentially have important implications for the transplantation of HLA-mismatched marrow in man. Other studies have examined the specificity of the T cells effecting in vivo graft rejection responses. Using allophenic mice as the donors of skin allografts, it was found that the effector mechanism of skin allograft rejection is mediated by T cells that assess individual cells in the dermis of the graft for expression of foreign histocompatibility antigens.

Other investigators in the Branch have focused their research efforts on the characterizing structure, regulation, and function of genes involved in the immune response. Previously, it was shown that the swine class I MHC multigene family contains only seven members. Recent physical mapping has established that all of the genes are linked and resident within 500 kb. Determination of the DNA sequence of five members of the family has shown that the sequence organization of the class I SLA genes is similar to that of class I genes in other species. Three sub-groups have been defined; the genes of one sub-group are expressed ubiquitously; a second sub-group defines a differentiation antigen; a third sub-group does not appear to be expressed at all. In order to investigate the molecular basis for the differential patterns of expression, transgenic mice containing distinct swine class I genes have been generated. For one gene, encoding a classical transplantation antigen, it has been demonstrated that the pattern of expression of the swine gene in the transgenic parallels that observed in situ.

The characterization of DNA sequence elements and cognate transacting factor associated with class I MHC genes has been undertaken. A series of negative and positive regulatory DNA sequence elements within the 5' flanking region of the classical transplantation antigen gene have been identified. In particular, a novel negative regulatory element, whose function requires the presence of an enhancer element has been described. This interaction is mediated by transacting factors; future studies will attempt to elucidate the molecular mechanism of this interaction.

Class I MHC expression is known to be induced by immunomodulators. Recent studies have not only demonstrated the ability of a foreign class I gene in a transgenic mouse to respond to interferon, but have mapped the interferon responsive element. Furthermore, other studies have demonstrated that ethanol,

at physiologically attainable levels, is able to act as an immunomodulator by elevating steady state RNA and cell surface levels.

Other studies focus on isolating and characterizing genes involved in the regulation of lymphocyte proliferation. Over 60 novel cDNA clones have been isolated which represent the immediate proliferative response of resting human peripheral blood T cells activated by mitogen. This primary response is highly complex, both in terms of the number of inducible genes as well as the diversity of their regulation. A significant number of the genes involved are restricted in their tissue specificity and thus may encode the differentiated functions of activated T cells. Surprisingly, many inducible genes are inhibited by the immunosuppressive drug cyclosporin A, which is known to repress the transcription of several lymphokine genes. In addition, a subset of inducible genes are expressed in HTLV-I infected T cell clones. Such abnormally-expressed genes may play a role in the deregulated growth of such cells.

The genetic regulatory mechanisms that govern tissue specific expression of the T cell receptor (TCR) β chain has been investigated utilizing an in vitro model of cell type specificity. Both transient and permanent transfection systems have been used to assay the transcription of a genomic TCR β chain gene in T cells, fibroblasts, and a variety of hematopoietic tumor cells. The results suggest the existence of a tissue-specific negative regulatory element. Future experiments will utilize fine structure analyses to identify the location of this element.

Previous studies demonstrated that human T and K lymphocytes, targeted against tumors by heterocrosslinked antibodies, are able to prevent the establishment and growth of human tumors. Whereas all of the targeted T cell cytotoxicity as measured in a ^{51}Cr-release assay resided in the CD8+, CD4- subset, most of the tumor neutralizing activity was in the CD4+ subset. An in vitro tumoristasis assay was developed to investigate this discrepancy further. Results from these studies show that targeted PBL exhibit strong tumoristatic activity, and that much of this activity resides in the CD4+ subset. Preliminary evidence suggests that targeted tumoristasis may be mediated through a soluble factor. Anti-CD3 augments the cytotoxic activity of murine cells activated by recombinant IL-2 and induces the generation of activated killer (CD3-AK) cells by triggering endogenous production of lymphokines. CD3-AK cells which react with a wide variety of tumor cells, grow longer in culture than LAK cells and thus may be an ideal candidate for use in adjuvant adoptive immunotherapy of cancer.

SUMMARY REPORT

LABORATORY OF GENETICS, DCBD, NCI

October 1, 1987 through September 30, 1988

General

In the last year members of the Laboratory of Genetics have organized four workshops, attended by N.I.H., U.S. and foreign scientists.

Beverly Mock organized a workshop on Immunoregulatory Genes, with emphasis on current advances in the fine structure mapping of genes relevant to the immune system in the mouse. This workshop was attended by leading mouse geneticists and is the third in a series that focus on genes relevant to immunogical functions and tumor formation in mice. This year we hosted the 6th workshop on Mechanisms in B-cell Neoplasia (which is held every other year at the Basel Institute of Immunology). This field is progressing in an exciting way and the meeting was enthusiastically received. Grace Shen-Ong organized a one day workshop on Mechanisms in Myeloid Tumorigenesis, which preceeded the B cell meeting. Experimental myeloid tumorigenesis, and its links to human tumors is now beginning a number of new develop in a promising way. Experimental model systems for studying the pathogenesis of myeloid tumors in mice are being developed. This meeting was thought to be quite timely, and we hope to continue this series. Sandra Smith-Gill organized a workshop on the immunology of lysozyme. This area of research focuses on the structure, and regulation of antiprotein antibodies.

Many of the projects in the Laboratory of Genetics deal with genes that play a role in neoplastic development, either as targets of mutagenesis (oncogenes) or as genes that modify the host response to tumorigenic agents. i.e. genes that determine susceptibility and resistance to tumor development.

A model system developed in the Laboratory of Genetics for testing the oncogenic activity of transforming retroviruses for hematopoietic tissues, has been the pristane conditioned adult BALB/cAn mouse. Oncogenic viruses are usually inactive in non-conditioned hosts, but pristane expands and prepares the target population. Mice develop myeloid or B-cell tumors.

1. Linda Wolff and Grace Shen-Ong have shown that the injection of Moloney virus (MoMuLV) into pristane conditioned mice induces monoblastic tumors, all of which contain MoMuLV insertions into the c-myb gene. This type of myeloid tumor development can be inhibited by the chronic administration of indomethacin in the drinking water. Myeloid tumors induced c-myc containing retrovectors are not inhibited by indomethacin. How this intriguing effect takes place is not known.

2. We continue to identify and characterize genes that determine resistance to plasmacytoma induction by pristane using BALB/cAn-DBA/2 congenic strains that have been constructed in the Laboratory of Genetics. Three genes have been identified by linkage Pctr-1 (distal end, chr4), Pctr-2 (linked to Ly-6, c-myc on chr15) and Pctr-3 (linked to Qa2 chr17).

We seek to precisely localize the genes and find their products. Potentially relevant closely linked genes have been identified Pctr-1 is the same as or closely linked to Rep-1 a gene whose product appears to determine efficient repair of x-ray induced damage to chromatin (in collaboration with K. Sanford); Pctr-2 is closely linked to a gene MUP-M1 (MUP-modifier-1) which modifies the production of the 4.1 component of MUP (with R. Duncan).

3. The RIM retrovector (which contains c-myc under the control of immunoglobulin promoters and enhancers) and v-Ha ras, induces plasmacytomas in 80 to 100% of BALB/cAn mice with short mean latent periods 60 days. RIM also induces plasmacytomas 80% of (BALB/c x DBA/2) F1 hybrids that are resistant to pristane induced plasmacytomas. B. Mock has found that plasmacytomas can also be induced in 45% [DBA/2 x (BALB/c x DBA/2 F1 hybrids)] but in only very low incidence in DBA/2 mice, indicating that susceptibility to plasmacytoma induction by Pristane and RIM virus is determined by a single gene of BALB/cAn origin. Work is underway to link, and isolate this gene.

4. The bcl-2 gene is an important gene that is implicated in the pathogenesis of follicular lymphomas in man but its made of action in lymphomas development is not understood. In the mouse F. Mushinski, and W. Davidson have found that bcl-2 is transcribed in a very narrow window of time in B-cell development i.e. the pre-B cell period.

5. The c-myb oncogene is very large and complex gene. Grace Shen-Ong has characterized alternative splicing of the c-myb gene in normal and tumor cells, and is seeking to determine if alternative transcription of this gene may be important in oncogenesis.

6. IL-6 has been shown to play an important role in the generation of primary immune responses to influenza virus hemmaglutinin but is not active in driving secondary responses (D. Hilbert, S. Rudikoff, R. Nordan).

7. The relationship of the myeloid and B-cell lineages to each other in normal hematopoiesis is not clearly understood. W. Davidson has characterized a series of cell lines that can apparently differentiate along either pathway, thus indicating the presence of a pivotal stem cell with potential to differentiate into macrophages or B cells.

8. The retroviral element SFFV induces a massive and dramatic erythroid proliferation in mice. S. Ruscetti has found that this is not due to the autocrine induction of erytropoietic (Epo) or a change in the number of Epo-receptors.

9. The X-ray structures of two monoclonal Fab-hen eggwhite-lysozyme complexes have been determined at high resolution. These model systems provide the basis for experiments on studying the role of specific amino acids in determing an epitope structure. Analysis of models such as these should permit understanding the structural basis of antibodies to proteins, and potentially pave the way to designing autobodies of desired specificity.

10. H. Coon has cultured 16 odorant-responsive cell lines from the rat olfactory epithelium, and has evidence that these are derived from neuroblasts and are probably immature neuron precursors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05596-19 LGN

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pathogenesis of plasma cell neoplasia: Resistance and susceptibility genes

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

P.I.:	M. Potter	Chief, Lab. of Genetics	LGN,NCI
	E.B. Mushinski	Bio. Lab. Technician	LGN,NCI
	L. Byrd, R. Duncan	Biologists	LGN,NCI
	K. Huppi	Staff Fellow	LGN,NCI
	B. Mock	Staff Fellow	LGN,NCI
	E. Shacter	Expert	LGN,NCI
	K. Sanford	Chief, In Vitro Carcin Sect.	LCMB,NCI

COOPERATING UNITS *(if any)*
Dr. H.C. Morse, III, NIAID; Dr. F. Wiener, Karolinska Institutet, Stockholm, Sweden; Dr. R. Parshad, Howard Univ., Wash. DC; Dr. K. Marcu, SUNY, Stony Brook, NY; Dr. L. Blankenhorn, Hahneman Medical School, Phil., PA

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
9.0	5.0	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.)*

The major project in the laboratory is to determine pathogenetic mechanisms involved in the development of paraffin oil (pristane) induced plasmacytomas in BALB/c mice. BALB/cAn mice are highly susceptible to developing these tumors while most other strains are resistant. Over 95% of plasmacytomas induced by pristane have chromosomal translocations [rcpt(12;15), rcpt(6;15)] involving directly or indirectly the c-*myc* locus on chr 15. We have developed model genetic systems for finding the genetic basis of susceptibility and resistance to plasmacytoma development by using BALB/cAn.DBA/2 (C.D2) and BALB/cAn.BALB/cJ (C.J) congenics. Three genes have been identified that confer partial resistance: Pctr-1 (*Fv-1*, chr 4), Pctr-2 (*Ly-6*, chr 15) and Pctr-3 (*Qa2*, chr 17). Congenic mice constructed by combining these genes have produced even stronger resistance. In collaboration with Dr. K. Sanford, we have found genes associated with DNA repair deficiencies in BALB/cAn mice and C.D2 congenics: Rep-1 (*Pep-3*, chr 1) and Rep-2 (*Fv-1*, chr 4). Rep-2 and Pctr-1 could be the same gene. The linkage of Pctr-2 to *Ly-6*, c-*myc* *Mupm*-1 (MUP modifier) and *Afr-1* (regulator of alpha fetoprotein) has been established.

Plasmacytomas can be induced with short latent periods in BALB/cAn mice by the infection of pristane conditioned mice with retroviruses containing oncogenes. Our recent studies demonstrate the potent cooperative action of *myc* and *ras* (in collaboration with K. Marcu, Stony Brook). (BALB/c x DBA/2)F_1 hybrids injected with RIM virus (containing c-*myc* and v-*Ha*-*ras*) and pristane are highly susceptible to plasmacytoma induction, indicating the existence of a dominant gene of BALB/c origin that confers susceptibility.

GPO 914-918

Genetics of susceptibility to plasmacytoma induction (with R. Duncan, K. Huppi, B. Mock)

Three BALB/c.DBA/2 congenic strains: C.D2 Fv-1 (chr 4), C.D2 Ly-6 (chr 15) and C.D2 Qa2 (chr 17) show partial resistance to plasmacytoma induction by i.p. pristane. Further, C.D2 Fv-1 + C.D2 Qa2 shows much stronger resistance indicating that resistance is in a given strain determined by multiple weak acting genes. Ongoing and future work is designed to identify the resistance genes located in the DBA/2 DNA segment carried by the congenic. In experiments with K. Huppi we have found that C.D2 Fv-1 N10 carries a large DNA segment, including Mtv-13. However, by C.D2 Fv-1 N19, the Mtv-13 gene has been lost. We are currently testing the N19 mice for plasmacytoma resistance (Pctr) to determine if Pctr-1 is still linked to Fv-1. The N19 mice also carry the Rep-2 gene. The order of genes on chr 15 near Ly-6 has been established by Huppi et al., and c-myc is now known to be ~ 2 cM from Ly-6. The origin of Festenstein's BALB/c stock was not clear. We, therefore, have backcrossed Ly-6 from Festenstein's C.D2 DAG onto BALB/cAnPt and are testing the N16 mice. The C.D2 Qa2 mice have been further advanced to N11. We are still searching for markers to facilitate the fine structure mapping of these regions, in preparation for establishing recombinants.

(BALB/c x DBA/2)F_1 hybrids are resistant to pristane induced plasmacytomagenesis, but highly susceptible to pristane-RIM virus induced plasmacytomagenesis. RIM virus which contains c-myc and ras oncogenes appears to bypass the requirement for the development of chromosomal rearrangements that are essential for myc gene activation. In experiments with B. Mock we have tested first generation backcross (to DBA/2) mice and found that the yield of plasmacytomas is approximately 50% of that in CDF_1, suggesting a single dominant gene of BALB/c origin determines susceptibility. Since the RIM virus system induces plasmacytomas with mean latent periods of 60 days, finding this susceptibility gene Pcts-1 should be considerably easier to locate. The preliminary indications are that the Pcts-1 is located on chr 15 (in collaboration with B. Mock). The mapping of genes proximal to c-myc on chr 15 has proven difficult due to the lack of markers. However, we (R. Duncan, E. Blankenhorn) have found two markers, Mupm-1 (MUP-modifier gene) and Afr-1 (regulator of alphafetoprotein) that are centromeric to c-myc. We are continuing to establish the order of genes on chr 15 and to find the product of Mupm-1.

In collaborative experiments with L. Byrd, it has been found that BALB/cAn nu/nu mice (BALB/c nude) are resistant to pristane but not RIM virus plasmacytomagenesis. Further, BALB/cAn nu/+ mice also have partial resistance. We are attempting to locate the genetic basis of this resistance. Experiments are in progress to determine the exact location of nu on chromosome 11.

Rapid induction of plasmacytomas

We have been developing over the last few years a rapid model of plasmacytoma induction by infecting pristane treated mice with retroviruses containing oncogenes. We have in the last year identified a new viral element constructed by K. Marcu that is superior to all others previously tested. This retrovirus (RIM) contains a V-Ha-ras gene (under the control of the viral LTR) and a mouse

c-myc gene under the control of the heavy chain enhancer and the V_H promoter. When this virus is given to mice that have received 0.5 ml of pristane, 80-90% of the mice develop plasmacytomas within 120 days, with a mean latent period of 60 days. Thus far, viruses that contain either of the oncogenes alone are relatively ineffective. A few tumors have been found with the virus containing the V-Ha-ras oncogene, but those all develop chr 15 translocations. The results indicate the need for cooperation of the 2 oncogenes and suggest the need to examine the role of ras genes in plasma cell tumors. The myc-ras tumors induced by the RIM virus express predominantly IgM paraproteins and have strong monoclonal bands on electrophoresis. The tumors grow rapidly and are not priming dependent. Further, we can induce these tumors in the genetically resistant $(BALB/c \times DBA/2)F_1$ mouse.

Effects of chronic indomethacin treatment on plasmacytomagenesis

Indomethacin administered in the drinking water at 20 µg/ml during the latent period of plasmacytomagenesis strongly inhibits plasmacytoma formation and the progression of foci. In collaborative experiments with E. Shacter we are trying to determine the relevant mode of action of indomethacin. Work nearing completion indicates indomethacin inhibits the formation of the potentially mutagenic HCLO radical in neutrophils. This is apparently accomplished by the inhibition of myeloperoxidase. This finding strengthens the hypothesis that the chronic inflammatory process initiated by intraperitoneal pristane generates radicals that are potential mutagens for associated B lymphocytes. Dr. Shacter is currently attempting to develop an in vitro cellular model for radical production, DNA damage and repair.

Emily Shacter - Summary of work:

The work has been directed toward answering the following questions:

(a) Do neutrophils and macrophages from plasmacytoma-susceptible and -resistant mice differ in their capacity to produce reactive oxygen intermediates (ROIs)?

(b) Can pristane-elicited peritoneal neutrophils and macrophages induce DNA damage in neighboring B lymphocytes?

(c) Do B lymphocytes from BALB/cAn mice differ from other mice in their ability to repair DNA strand breaks detectable in a biochemical assay?

(d) What effect, if any, does indomethacin have on the above processes?

(e) Can we establish a tissue culture model with RIM virus-induced tumors to study the phenotypes of the mice in which they were induced?

Major Findings:

1. Extensive studies on pristane-elicited neutrophils and macrophages from BALB/cAn, DBA/2, CDF_1, and BALB/cJax mice have shown that they have nearly equal capacities to produce O_2^-, H_2O_2, and HOCl upon stimulation with phorbol myristate acetate or with opsonized zymosan.

2. By employing the alkaline elution assay to study cellular DNA damage and repair, we have found that pritane-elicited neutrophils from both BALB/cAn and CDF_1 mice can cause extensive and prolonged DNA damage in neighboring plasmacytoma cells and that H_2O_2 is an essential mediator in this process. Indomethacin is ineffective at inhibiting acute formation of single strand breaks.

3. LPS-blasts prepared form BALB/cAn, CDF_1, and DBA/2 mice repair single strand DNA breaks induced by H_2O_2 or low doses of γ-irradiation at roughly equal rates. However, they appear to differ in their capacity to repair double strand DNA breaks induced by high doses of γ-irradiation (neutral elution assay); cells from BALB/cAn are slower than those from the resistant strains.

4. Cell lines were established from pristane/RIM virus-induced tumors from BALB/cAn and CDF_1 mice. The cells are efficient in repairing DNA strand breaks and appear to mimic the DNA repair phenotypes of the mice in which they were induced. Hence, these cell lines may provide an in vitro model in which to study the biochemical genetics of plasmacytomagenesis.

Publications:

Clynes R, Wax J, Stanton LW, Smith-Gill S, Potter M, Marcu KB. Rapid induction of IgM secreting murine plasmacytomas by pristane and an IgH promoter-enhancer driven c-myc/v-Ha-ras retrovirus. PNAS USA, in press.

Blankenhorn EP, Duncan R, Huppi K, Potter M. Chromosomal location of the regulator of alpha-fetoprotein, Afr-1. Genetics, in press.

Potter M, Sanford, KK, Parshad R, Tarone RE, Price FM, Mock B, Huppi K. Genes on chromosomes 1 and 4 in the mouse are associated with repair of radiation-induced chromatin damage. Genomics, in press.

Duncan R, Matthai R, Huppi K, Roderick T, Potter M. Genes that modify expression of major urinary proteins in mice. J Mol Cell Biol 1988;8.

Huppi K, Duncan R, Potter M. Myc-1 is centromeric to the linkage group Ly-6--Sis--Gdc-1 on mouse chromosome 15. Immunogenetics 1988;27:215-19.

Shacter E, Chock PB. Covalent interconversion and thermostability of proteins. In: Henle KJ, ed. Thermotolerance, Volume II: Mechanisms of Heat Resistance. Boca Raton: CRC Press, 1987;105-18.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08727-11 LGN

PERIOD COVERED
October 1, 1987 to September 30, 1988

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, laboratory, and institute affiliation)*

P.I.:	J.F. Mushinski	Medical Director	LGN, NCI
	G.L.C. Shen-Ong	Senior Staff Fellow	LGN, NCI
	K. Huppi	Staff Fellow	LGN, NCI
	D. Kunimoto	Guest Researcher	LGN, NCI
	A. McDonald	IRTA	LGN, NCI
	L. Miribel	Visiting Fellow	LGN, NCI
	J. Kurie	Biotech. Training Fel.	LGN, NCI

COOPERATING UNITS *(if any)*
D. Givol, Rorer Biotechnology, Inc.; K. Marcu, Dept. of Biochemistry, SUNY, Stony Brook, NY; H.C. Morse, III, LVD,NIAID; J. Ashwell, BRMF, NCI; F.D. Finkelman, Dept. of Medicine, USUHS

LAB/BRANCH
Laboratory of Genetics

SECTION
Oncogene

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	7	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 overall goal of this research is to study the activation of particular genes in diseased and normal cells in order to understand which genes may play important roles in the development of malignancies, autoimmune diseases and normal differentiation. The immune system has been chosen as the central focus of this research, and we have concentrated on the expression of "oncogenes," especially myc, myb, ras and bcl-2, as well as immunoglobulin and T cell receptor genes. To this end we have been studying the lymphoid tumors (particularly the plasmacytomas) that are regularly induced in BALB/cAnN mice by intraperitoneal injections of alkane mineral oils, such as pristane. These tumors represent immortalized lines of B lymphocytes or myeloid cells at different stages of differentiation. Currently we are using this model system of tumors to learn how the genes involved in myeloid and B cell carcinogenesis are organized and regulated. The activation of the myc oncogene has been clearly shown to be essential to the induction of plasma cell tumors, but it is likely that other genes must also be activated to complete the neoplastic transformation. The ras family of oncogenes is a likely candidate and we have evidence that mutations in one or more of these genes can be found in plasmacytomas and lymphosarcomas. The role these play in generation of these tumors is being studied. The myb gene is expressed in most hematopoietic tumors, and in certain virus-induced myeloid malignancies it is activated by insertion of a virus within the coding region. The regulation of this gene's expression is being investigated, and one example of alternative splicing of an extra coding exon has been found in normal and malignant cells. The putative proto-oncogene, bcl-2, appears to be expressed in a differentiation stage-specific and cell type-specific manner. Whether excess bcl-2 expression is tumorigenic in mice is being tested using retroviral constructs.

Major Findings:

a. Expression of oncogenes in normal and neoplastic B-lymphocytes. We found that expression of the nuclear oncogenes c-myc, c-myb and c-fos, was highest in tumors of pre-B lymphocytes and that it diminished in tumors of more mature B cells. The opposite was found for expression of Ha-ras. A unique pattern of expression was found for bcl-2 in that very early progenitor B cells (Pro B cells) such as HAFTL-1 and HAFTL-3 and very late plasmablasts and plasmacytomas contained no bcl-2 mRNA, while pre B and mature B cells did contain bcl-2 transcripts. This developmental regulation of bcl-2 expression in B-lymphocytes was confirmed in normal spleen cells which had been stimulated by injection of anti-IgD antiserum to divide and then mature into antibody-secreting cells. At the time antibody synthesis was maximal, the bcl-2 RNA levels were minimal or non-existent.

It seems that the regulation of bcl-2 expression in this system occurs primarily at the transcriptional level, but preliminary evidence suggests that post-transcriptional control may also be possible. Myeloid tumors seem to express bcl-2 abundantly as do T cell lymphomas, so we are presently studying whether bcl-2 is frequently down-regulated when these and other cell types are induced to differentiate.

b. Increased v-myc expression can induce murine lymphoid and myeloid tumors. Constitutive expression of c-myc is a consistent finding in pristane-primed BALB/c plasmacytomas and is usually a consequence of chromosomal translocation involving the c-myc locus. The requirement for the translocations can be replaced by retroviruses that express v-myc or c-myc, which verifies the essential role myc expression plays in induction of such tumors. Myeloid tumors are also induced by most myc retroviral constructs. The virus-induced lymphoid and myeloid tumors arise earlier in pristane-treated mice than such tumors appear in the absence of the viruses, presumably due to the immediate production of high c-myc levels caused by the virus. Careful study of DNA from successive generations of several myeloid and B-lymphocytic tumors induced by J2, J3 and J5 viruses which contain different combinations of v-myc and v-raf indicates that these two oncogenes act synergistically to induce tumors rapidly and often multiple tumors at the same time. The mixed tumors generally appear monoclonal after transplantation during which time the most vigorous tumor establishes dominance. Clonal dominance can change, however, as viral reintegration sometimes occurs and other factors, such as freezing and thawing in storage, can lead to selective loss of certain populations.

The manifold factors operating in vivo reflect the complex nature of tumor biology but the changes in clonal dominance may be more easily understood by studying successive generations of tissue cultured tumors. Helper-free tumor induction is also being initiated with J3 viruses to assess the role of viral reintegration in the progression of these tumors.

c. Control of myc expression. Deregulation of myc expression appears to be one of the key elements in plasmacytomagenesis, and up regulation of c-myc expression is well known to accompany stimulation of cell growth. Thus control of c-myc expression in normal and neoplastic cells continues to be a major

interest. Two plasma cell tumors, TEPC1165 and TEPC2027, contain unusually high levels of c-myc mRNA. These high levels have been shown to result from a combination of increased transcription (unusual in tumors) and increased stability of the RNA transcripts. These tumors have been useful to pin down a major factor that increases myc mRNA stability--the presence of unspliced intron 1 sequences in the c-myc mRNAs caused by mutations at the splice donor site. Sequence studies have revealed hitherto undetected point mutations in myc intron 1 in these two tumors, so we are now widening our DNA sequencing study of c-myc to look for mutations in this gene in other plasma cell tumors, including some with no karyologic abnormalities. It is hoped that DNA sequence studies upstream of c-myc exon 1 may reveal some clues to the mechanism responsible for increased c-myc transcription in these plasmacytomas.

Virus-mediated or transgenically-induced myc overexpression appears to be able to bypass some of the resistance to pristane-induced plasmacytomagenesis in non-BALB/c mice. Thus the myc locus of BALB/c or the proteins that interact with it must be important in conferring on this strain its unique susceptibility to plasmacytoma induction. Thus we have also undertaken the study of the fine structure and DNA sequence of the normal BALB/c, BALB/Jax and DBA/2 myc genes. We also will characterize and compare the nuclear proteins that interact with this gene in the susceptible and resistant strains.

d. Involvement of myb in tumorigenesis (Grace Shen-Ong, Dennis Kunimoto). We have previously identified a group of Abelson virus-induced myeloid tumors (ABMLs) which carries a disrupted myb allele as a result of integration of the helper Moloney murine leukemia virus (M-MuLV). Almost all the ABMLs do not contain the acute transforming Abelson-MuLV. It was therefore not clear if the v-abl oncogene plays any role in the tumorigenesis of the ABMLs. In collaboration with Linda Wolff (Lab. of Genetics, NCI), we showed that M-MuLV alone is sufficient in inducing ABML-like myeloid tumors in pristane-treated BALB/c mice. The disruption of the myb locus by M-MuLV insertion in these tumors is the same as that in the ABMLs, again indicating that it is the altered c-myb and not v-abl that plays the important role in the induction of these tumors.

The M-MuLV insertion into the myb locus results in the expression of chimeric viral-myb transcripts as revealed by cDNA cloning and sequencing analyses. One of the ABML cDNA clones contains an additional in-frame internal myb exon (E6A). It has been suggested by another laboratory that the additional exon is due to internal alternate splicing solely as a consequence of the M-MuLV inserted upstream. However, based on RNA blotting experiments and RNAase protection studies, we demonstrated that the E6A exon is found in a minor population of myb transcripts in both normal and tumor cells. Although alternative splicing has been demonstrated in genes that are important in development, this is the first time the c-myb gene has been shown to express alternatively spliced transcripts. This supports a previous notion that regulation of c-myb expression could play a major role in proliferation and differentiation of cells during hematopoiesis. We have more recently identified additional alternatively spliced human and mouse myb cDNA clones, and we are currently studying these clones in order to gain more understanding of the molecular mechanisms underlying regulation of myb gene expression during normal hematopoiesis and tumorigenesis.

e. Ras mutations in B-lymphocytic tumors (Konrad Huppi, Laurent Miribel). Recent studies with retroviral constructs clearly indicate that oncogenic forms of c-myc, c-ras or c-abl can cooperate in the formation of B cell tumors. In view of the nearly universal involvement of the c-myc gene in mouse plasmacytomas we have begun to investigate the structure of endogenous ras and abl genes in these tumors. Genomic and cDNA clones encoding mouse H-ras, K-ras, N-ras and c-abl genes have been isolated and sequenced from normal and tumor libraries.

Since the transforming mutations in ras genes characteristically occur in codons 11-13 or 59-61, riboprobes and specific oligonucleotide probes spanning these codons were constructed and utilized to detect point mutations by RNase mismatch or Southern hybridization analysis, respectively. These studies, in concert with restriction endonuclease analysis or direct cloning and sequencing indicate that late stage B cell tumors such as mouse plasmacytomas rarely contain point mutation of ras genes. In one instance, a pristane-induced plasmacytoma, TEPC1174, was determined to carry a single amino acid substitution (Leu → Arg) at residue 23 of K-ras. We are investigating whether this novel alteration (specifically the acquisition of an Arginine at codon 23) is sufficient to induce transformation in NIH 3T3 cells. Since point mutations in ras genes do not appear to be frequent in plasmacytomas, we are investigating whether changes in ras expression, such as alternative splicing of exons (i.e., upstream) or selective amplification of normal ras alleles may be a significant step in tumor formation.

Preliminary experiments indicate that N-ras is frequently mutated at codon 12 or 59 in a group of Abelson-virus induced pre-B lymphosarcomas. Genomic cloning and sequencing studies are underway to confirm these mutations. It is not yet known whether these mutations are an integral part of the initial transformation event or accumulate subsequent to it. Thus future studies will determine if these mutations are present in early generation tumors and if they persist during subsequent transplantation.

Publications:

Gurfinkel N, Unger T, Givol D, Mushinski JF. Expression of bcl-2 gene in mouse B lymphocytic cell lines is differentiation stage specific. Eur J Immunol 1987; 17:567-70.

Mock B, Skurla R, Huppi K, D'Hoostelaere L, Klinman D, Mushinski JF. A restriction fragment length polymorphism at the murine c-myb locus. Nucleic Acids Res 1987;15:4700.

Mushinski JF, Davidson WD, Morse HC. Oncogene activation in mouse leukemias-lymphomas: spontaneous and induced oncogene expression in murine B lymphocytic neoplasms. Cancer Invest 1987;5:345-68.

Mountz JD, Smith J, Snapper CM, Mushinski JF, Finkelman FD. Polyclonal activation of the murine immune system by an antibody to IgD. VIII. Stimulation of IgD secretion. J Immunol 1987;139:2172-8.

Heller M, Owens JD, Mushinski JF, Rudikoff S. Amino acids at the site of V_K-J_K recombination not encoded by germline sequences. J Exp Med 1987;166: 637-46.

Mock BA, Givol D, D'Hoostelaere LA, Huppi K, Gurfinkel N, Unger T, Potter M, Mushinski JF. Mapping of the bcl-2 oncogene on mouse chromosome 1. Cytogenet Cell Genet 1988;47:11-5.

Sneller MC, Kunimoto DY, Mushinski JF, Strober W. Molecular analysis of membrane γ 2b heavy chain expression. Molec Immunol 1988;25:393-401.

Shen-Ong GLC, Wolff L. Moloney murine leukemia virus-induced myeloid tumors in adult BALB/c mice. Requirement of c-myb activation but lack of v-abl involvement. J Virol 1987;61:3721-5.

Shen-Ong GLC. Alternative internal splicing in c-myb RNAs occurs commonly in normal and tumor cells. EMBO J 1987;6:4035-9.

Gardner SM, Mock BA, Hilgers J, Huppi KE, Roeder WD. Mouse lymphotoxin and tumor necrosis factor: Structural analysis of the cloned genes, physical linkage and chromosomal position. J Immunol 1987;139:476-83.

Huppi KE, D'Hoostelaere LA, Mock BA, Jouvin-Marche E, Behlke MA, Chou HS, Berry RJ, Loh DY. T cell receptor $V_T\beta$ genes in natural populations of mice. Immunogenetics 1988;27:51-6.

Huppi K, Duncan R, Potter M. Myc-1 is centromeric to the linkage group Ly-6--Sis--Gdc-1 on mouse chromosome 15. Immunogenetics 1988;27:215-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05553-19 LGN

PERIOD COVERED
October 1, 1987 to September 30, 1988

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
	W. Davidson	Visiting Associate	LGN, NCI
	R. Nordan	Staff Fellow	LGN, NCI
	J. Hyde	Postdoctoral Fellow	LGN, NCI
	A. Cuddihy	Graduate Student	LGN, NCI
	D. Hilbert	Guest Researcher	LGN, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.0	5.0	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.)*

1) A series of experiments have been performed to investigate the biological effects of murine interleukin-6 (IL-6). Initial studies have demonstrated that this factor may be involved in both B- and T-cell development. IL-6 enhances proliferation of thymocytes treated with lectin and IL-1. A similar enhancement is observed with IL-4, and combinations of these factors have begun to define a hierarchy of action for three well-characterized cytokines.

The role of IL-6 in B-cell development has been examined in the immune response to influenze hemmagglutinin. Antisera to IL-6 completely inhibit the primary response to this antigen and the inhibition is reversed by the addition of purified IL-6. In contrast, the antisera has no effect on the secondary response indicating an obligatory requirement for this factor in the early stages of the B-cell response and a differential responsiveness of primary versus secondary cells.

2) Studies have been initiated to identify genes and proteins associated with cell differentiation. Clonal cell lines which can be induced to differentiate are being used to construct subtractive cDNA libraries for gene isolation. Nuclear matrix preparations are simultaneously being analyzed to determine the magnitude of expressed protein differences between successive developmental stages and to potentially isolate specific proteins.

A. Cell Growth and Differentiation

In the preceding annual report we described a major shift in the direction of this laboratory into the area of cell growth and differentiation. Development of systems to study aspects of these phenomena has occupied the major effort of the lab for the past year and initial results obtained are described below.

1. We have previously identified a factor produced by macrophage lines which is required for the growth of a number of plasmactyomas in vitro. This factor has been purified to homogeneity and a partial amino acid sequence determined. Based on the protein sequence, oligonucleotide probes were constructed and attempts made to clone the corresponding gene. During the course of these experiments, the same gene was cloned by a number of other groups resulting in its identification as the murine form of interleukin-6 (IL-6). We have therefore proceeded with studies to evaluate the biological functions(s) of this molecule.

A polyclonal rabbit antisera has been prepared to IL-6 and employed to assess the role of this cytokine in lymphocyte development. IL-6 appears to act on both T and B-cells and may be important in the ontological development of both cell lineages. Thymocytes initially treated with lectin and IL-1 undergo enhanced proliferation upon the addition of IL-6 whereas IL-6 alone has no effect. A similar effect is seen with the lymphokine IL-4. The enhanced proliferation induced by IL-4 is inhibited by antisera to IL-6 and the IL-6 induced enhancement is similarly inhibited by monoclonal antibodies to IL-4 indicating that either both of these factors act simultaneously on the same subpopulation or on different subpopulations which must then interact to effect enhanced proliferation. While such alternatives remain to be clarified, these experiments begin to establish a hierarchy of action for three known lymphokines (IL-1, 4, 6) in the induction of thymocyte proliferation. Experiments are currently being initiated using IL-6 riboprobes to demonstrate in situ expression in the thymus and other centers of lymphoid proliferation to provide a potential in vivo correlate to the present observations.

The initial observation of plasmacytoma dependence on IL-6 suggested a potential role in B-cell development. This hypothesis has been tested by examination of the ability of anti-IL-6 to inhibit primary and secondary antigen specific B-cell responses. Results of these studies demonstrate that the primary response to influenza hemagglutinin is completely inhibited by this antisera and that the inhibition reversed by addition of purified IL-6. In contrast, antisera to IL-6 has no effect on the secondary response of B-cells previously exposed to antigen. There is, therefore, an obligatory requirement for IL-6 in the early stages of the B-cell response to antigen, presenting the first instance in which a known lymphokine differentiates between primary and secondary B-cells in terms of biological effect.

As an extension of earlier studies demonstrating in vitro plasmacytoma dependence on IL-6, we are attempting to evaluate the role of IL-6 in vivo. These studies will involve a regimen of treatment of recipients with anti-

IL-6 during tumor challenge. Such experiments would optimally be performed using highly inhibitory monoclonal antibodies which, as yet, are not available. We are in the process of attempting to generate such antibodies, but, in the mean time, preliminary experiments will be conducted with available rabbit antisera.

An additional aspect of IL-6 biology is being approached in collaboration with Steve Rosenberg's lab. The observation has been made that treatment of mice or patients with tumor necrosis factor (TNF) can result in significant tumor reduction although pronounced side effects are encountered. We have observed that following such treatment serum levels of IL-6 are drastically elevated suggesting a possible role of Il-6 in the observed toxicity. Experiments are currently underway to further define the role of IL-6 in these circumstances and to evaluate the possible sue of anti-IL-6 in the inhibition of toxic effects.

2. A second series of experiments designed to study regulation of differentiation consists of the detailed characterization of clonal cell lines which can be induced to further differentiate. One such system involves a pre-B cell line which upon growth in the presence of bacterial lipopolysaccharide can produce fully functional macrophages. Analysis of these lines is described in detail in the accompanying report by Wendy Davidson. Other lines being examined are B-ells which can be induced to either express or secrete immunoglobulin. One of the primary aims of these studies is to generate subtractive cDNA libraries for the isolation of gene specifically involved in the given differentiation pathway. Nuclear matrix extracts are also being made simultaneously to analyze by two dimensional electrophoresis the differences in protein expression associated with the respective stages.

B. Genetic regulation of lymphoid and myeloid cell differentiation and function (W. Davidson)

Summary

1) A longstanding interest in our laboratory has been the study of early B lymphocyte differentiation and the possible developmental relationship between certain B lineage cells and cells of the monocyte/macrophage lineage. The existence of a bipotential progenitor cell was suggested by our early studies of tumor cells and reports of patients with clonally related lymphocytic and myelomonocytic leukemias. Our initial approach to this question was to identify transformed haematopoietic progenitor cells with the potential to differentiate along either the B lymphocyte or macrophage pathways. Two progenitors exhibiting these characteristics and their differentiated progeny have been isolated and characterized for cell surface phenotype, oncogene expression, lymphokine production and function. The availability of these lines offers a unique opportunity to isolate and study genes that: 1) regulate the commitment of cells to a particular lineage, and 2) are involved in lineage-restricted cell functions. The identification of these two categories of genes will be the central focus of our future studies. Subtractive cDNA libraries between progenitor cells and macrophages are being constructed for this purpose.

2) A second major interest is the role of individual genes in the development of autoimmune disease in mice. Two genes lpr and gld have been studied extensively. The results showed that both genes cause remarkably similar disease in C3H mice characterized by the expansion of an unusual population of T cells that lack Ly-2 or L3T4 antigens but express the α and β chains of the TcR for antigen and all components of the associated T3 complex. Interestingly, the cells also express high levels of the B cell antigens Ly-5 (B220) and PC.1. Although the cells appear to express a normal TcR complex they are functionally inert and cannot be induced to produce IL-2, IL-3 or γ-IFN. Additional studies showed that the p21 molecule of the T3 complex was constitutively phosphorylated a characteristic usually associated with cell activation. Recent studies of the abnormal T cell population in lpr and gld mice have focussed on two topics: 1) the abnormality in signal transduction and 2) a possible role for lymphokines in lymphoproliferation either through an autocrine mechanism or from an exogenous source.

Cloning and Characterization of Cells to be used in Studies of Genes Controlling Cellular Differentiation and function

Studies of the cell surface phenotype and Ig heavy chain gene rearrangement of a panel of cloned cell lines derived by in vitro transformation of fetal liver and bone marrow cells with a variety of oncogenes revealed three cell lines of interest in the context of the current project. Two of these, HAFTL-3 and BAMC1, had the hallmarks of early B cell precursors and differentiated spontaneously into macrophagelike cells. A third line, HAFTL-1, had the features of a more immature B cell progenitor and could be induced to differentiate following stimulation with LPS into cells with either a more mature B cell progenitor and could be induced to differentiate following stimulation with LPS into cells with either a more mature B cell phenotype or cells with the characteristics of monocytes and macrophages. HAFTL-1 and HAFTL-3 progenitors and differentiated progeny were characterized extensively as outlined below.

Phenotype. Both parental lines were Ly-1$^+$, FcR$^+$, expressed the B-lineage restricted markers Lyb-2, Lyb-8 and Ly-5(B220) but were Ia$^-$, ThB$^-$, sIg$^-$ and Mac-1$^-$. By contrast, the macrophage lines derived from these progenitors continued to express Ly-1 and FcR, acquired Mac-1, Mac-2 and Ia and ceased to express Lyb-2, Lyb-8 and Ly-5(B220). The continuous expression of Ly-1 on the macrophage lines suggested to us that a common progenitor may exist that can give rise to Ly-1$^+$ B cells and a closely related population of Ly-1$^+$ macrophages. To determine whether Ly-1$^+$ monocytes and macrophages exist in normal animals, we have prepared reagents that will allow us to simultaneously analyze cells for sIgM, Mac-1 and Ly-1 expression by three colour flow microfluorometry.

Gene transcription. Northern blot analysis of poly A$^+$ mRNA revealed significant differences between the lines. Both progenitor populations expressed sterile IgM transcripts, an Ly-5 transcript corresponding in size to that encoding the B cell isoform of Ly-5, Ly-5 (B220) and transcripts for λ5 and Vpre-B1 two genes expressed exclusively in pre-B cells. No mRNA transcripts for GM-CSF or IL-6 were detected. By comparison, HAFTL-1 and HAFTL-3 macrophage lines had

no detectable IgM, λ5 or $V_{pre-B}1$ transcripts but expressed both GM-CSF and IL-6 mRNA. In addition, the Ly-5(B220) mRNA species was substituted with a lower molecular weight mRNA thought to encode the macrophage isoform of Ly-5.

Lymphokine Production. Both IL-6 and GM-CSF have been shown to play important roles in haematopoiesis. It was possible, therefore, that the production of IL-6 and GM-CSF by macrophage lines may lead to the recruitment of macrophages from the progenitor population. Alternatively, the progenitor cells may secrete small amounts of lymphokine that result in self-induced differentiation. These questions were approached using the HAFTL-1 system. First, we confirmed using bioassays that the macrophage lines but not the progenitor line produced IL-6 and GM-CSF. Interestingly, stimulation of HAFTL-1 with LPS resulted in the production of high levels of GM-CSF and low levels of IL-6. LPS treatment of a number of other cell lines gave similar results and demonstrated for the first time that pre-B cell lines can produce both lymphokines. It is also notable that LPS-stimulated HAFTL-1 cells producing high levels of GM-CSF had very low levels of GM-CSF mRNA that could be stabilized by treatment of the cells with cycloheximide. By contrast the macrophage lines had significantly higher levels of GM-CSF m-RNA. These differences in steady state mRNA levels suggest that the GM-CSF gene may be differentially regulated in B lineage and macrophage cells.

In other preliminary experiments we were unable to accelerate myeloid differentiation of HAFTL-1 cells by the addition of recombinant GM-CSF or highly purified IL-6. Further experiments are proposed using limiting dilution cultures and various combinations of lymphokines (IL-6, GM-CSF, G-CSF, M-CSF and IL-3).

Function. The HAFTL-1 and HAFTL-3 macrophage lines exclusively produced high levels of non-specific esterase and lysozyme, were phagocytic and able to present antigen to B cells.

In summary, our studies have demonstrated the existence of cells with the hallmarks of $Ly\text{-}1^+$ B lineage progenitors that can differentiate into fully funtional stable macrophages. Differentiation was shown to be associated with the down regulation of B-lineage restricted gene expression and the induction of macrophage-related genes. The extensive characterization of these lines has laid the foundation for future molecular studies.

Construction of cDNA libraries. To initiate our studies of differentiation at the molecular level, we chose to use HAFTL-1 and one of its macrophage derivatives. After some technical difficulties we have now constructed cDNA expression libraries and are in the process of characterizing them. Ultimately, cDNA subtractive libraries will be used to isolate progenitor and macrophage specific cDNA clones. Although our primary interest is the identification of genes that regulate differentiation, our system also offers the opportunity to study the regulation of transcription of the IgH, λ5, $V_{pre-B}1$, GM-CSF and IL-6 loci as well as the regulation of expression of lineage-specific isoforms of Ly-5. The macrophage libraries will also be useful for the identification of macrophage-specific cell surface markers since very few of these have been identified in the mouse.

Further Characterization of the Abnormal T Cell Population Expanded in C3H-lpr/lpr and C3H-gld/gld Mice. C3H mice homozygous for the lpr or gld mutant genes develop remarkably similar disease characterized by the development of massive lymphadenopathy and the production of autoantibodies. Lymphadenopathy results from the expansion of an unsual, relatively homogeneous population of cells that express an apparently normal TcR receptor complex but neither L3T4 nor Ly-2. An additonal peculiarity is the high level of expression of two B cell-associated antigens Ly-5(B220) and PC-1. Although the cells express all the components of the TcR complex they are functionally inert and unable to produce lymphokines such as IL-2, γ IFN and IL-3. The inability to respond to exogenous stimuli led us to predict that lpr and gld abnormal T cells may exhibit defects in receptor signal transduction. The cells therefore were examined for their ability to phosphorylate components of the T3 complex following stimulation with PMA or mitogen. The results showed that the p21 molecule of the T3 complex is constitutively phosphorylated on a tyrosine residue. The observation that a biochemical pathway identified with receptor activation is constitutively activated in gld and lpr abnormal T cells may explain the accumulation of these cells in lymphoid organs, their unusual phenotype and their inability to respond to exogenous stimuli.

We have recently undertaken studies to determine whether the abnormal T cell population could be stimulated by cross-linking the T3 complex or surface Thy-1 antigen with antibodies. Purified normal T cells and abnormal lpr and gld L3T4$^-$, Ly-2$^-$ T cells were compared for their responsiveness to stimulation with anti-T3 and anti-Thy-1 monoclonal antibodies alone or together with PMA, conA or IL-2. No significant proliferation was observed with the abnormal T cell subset providing further evidence that signal transduction across the membrane via a number of routes is substantially blocked in these cells. A final set of experiments is in progress to determine if the abnormal cells can proliferate in response to PMA and ionomycin.

An alternative explanation for the outgrowth of lpr and gld T cells and their refractoriness to exogenous stimuli is that they are perpetually activated by a growth factor or factors produced endogenously or possibly by another cell population. To address the question of autocrine growth factor production we are examining purified abnormal gld and lpr T cells for: 1) the presence of mRNA specific for lymphokines (IL-4, IL-5, IL-6, GM-CSF, M-CSF, TNF and neuroleukin), 2) spontaneous production of lymphokines and 3) induction of lymnphokine production. Preliminary results showed that both lpr and gld abnormal T cells spontaneously produce significant levels of neuroleukin (glucose-6-phosphate isomerase) mRNA. This mRNA species is not detected in normal resting T cells but is significantly elevated in lectinstimulated T cells. This observation provides further support for the suggestion that lpr and gld abnormal T cells are chronically activated. Experiments are planned to test the abnormal T cells for neuroleukin production and the effects of antineuroleukin antibodies on their growth and function.

Publications:

Davidson WF, Pierce JH, Rudikoff S, Morse HC III. Relationships between B cell and myeloid differentiation: studies with a B lymphocyte progenitor line, HAFTL-1. J Exp Med 1988;in press.

Mushinski JF, Davidson WF, Morse HC III. Activation of cellular oncogenes in human and mouse leukemia-lymphomas: Spontaneous and induced oncogene expression in murine B lymphocytic neoplasms. Cancer Invest 1987;5(4):345-68.

Trenn G, Takayama H, Davidson WF, Morse HC III, Sitovsky MV. Organization of lymphocyte plasma membrane. Surface protein-membrane matrix interactions in B cell lines at different stages of differentiation. Cell Diff 1988;22:233-44.

Mushinski JF, Mountz JD, Pierce JH, Pumphrey JG, Skurla RM, Finkelman FD, Givol D, Davidson WF. Expression of the murine proto-oncogene bcl-2 is stage specific and cell-type specific. Curr Top Microbiol Immunol 1980;in press.

Davidson WF. Mechansism of B lineage growth and differentiation-points of decision. In: Melchers F and Potter M, eds. Mechanisms of B cell Neoplasia. Basel: Editions Roches, 1980;12-23.

Jouvin-Marche E, Heller M, Rudikoff S. Gene correction in the evolution of the T cell receptor β chain. J Exp Med 1986;164:2083-8.

Majumdar A, Rudikoff S, Adhya S. Purification and properties of gel repressor pL-galR fusion in pkC31 plasmid vector. J Biol Chem 1987;262:2326-31.

Nordan RP, Neckers LM, Rudikoff S, Potter M. A growth factor required by plasmacytoma cells in vitro. Curr Top Microbiol Immunol 1986;132:114-20.

Kang CY, Cheng H, Rudikoff S, Kohler H. Idiotypics self binding of a dominant germline idiotype (T15): Autobody activity is affected by antibody valence. J Exp Med 1987;165:1332-43.

Simons SS, Pumphrey JG, Rudikoff S, Eisen HJ. Selective covalent labeling of cysteinen-656 of HTC cell gluocorticoid receptors by dexamethasone 21-mesylate. J Biol Chem 1987;262:9676-80.

Nordan RP, Pumphrey JG, Rudikoff S. Purification and NH_2-terminal sequence of a plasmacytoma growth factor derived from the murine macrophage cell line, P388D1. J Immunol 1987;139:813-7.

Heller M, Owens JD, Mushinski JF, Rudikoff S. Amino acids at the site of V_K-J_K recombination not encoded by germline sequences. J Exp Med 1987;166:637-46.

Singer DS, Hare J, Golding H, Flaherty L, Rudikoff S. Identification and characterization of a new sub-family of class I genes in the H-2 complex of the mouse. Immunogenetics; in press.

Osbourne BA, Golde TE, Schwartz, RL, Rudikoff S. Evolution of the IgA heavy chain gene in the genus Mus. Genetics; in press.

Greene WC, Depper JM, Crabtree GR, Rudikoff S, Pumphrey J, Robb RJ, Kronke M, Svetlik P, Peffer NJ, Waldmann TA, Leonard W. Molecular cloning of cDNA for the human interleukin-2 receptor. Hamatol Bluttrans fus 1986;29:388-96.

Singer DS, Ehrlich R, Satz L, Frels W, Bluestone J, Hodes R, Rudikoff S. Vet Immunol Immunopathol 1987;17:211-21.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05552-19 LGN

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
F. Curcio	Visiting Scientist	LGN, NCI
R. Swerdlow	Staff Fellow	LGN, NCI

COOPERATING UNITS *(if any)*

Dr. M. Luisa Brandi and Dr. K. Sakaguchi, NIDDKD, MD; Dr. F. Saverio Ambesi-Impiombato, Istituto di Patologia Generale, Naples, Italy

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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

X 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. We are now pursuing intensively a single cell system: culture of cells from the neonatal rat olfactory epithelium (OLFE). The projects involveing thyroid gland reconstruction and thyroid cell genetics have been postponed. We hope to continue these timely experiments as soon as our principal collaborator, DR. F. S. Ambessi, can return to the laboratory. Meanwhile, our progress with the OLFE has been sufficiently exciting during the past year that it has merited our full attention. Using complex media and substrates we have succeeded in culturing several cell types from the OLFE. The mixed, mass cultures of these cells provide an appropriate conditioned medium that has permitted the isolation of 20 clonal cell strains from 3rd to 6th passage cultures. We have shown that several of our cloned cell lines have sensitive (submicromolar) and selective (different response patterns in each line) odorant-dependent second messenger responses (both cAMP and Ca^{H}). This fact coupled with our demonstration that these same cell strains are positive for neuron-specific enolase now establish that we have right cell type in culture. We have continued the hybridoma screen using OLFE as antigen and fluorescent anti-mouse IgG staining of frozen sections of OLFE for selection. Development of this system would make available the first mammalian neuroblast to neuron cell culture system and provide a means to study the growth and differntiation dichotomy common to all blast cell systems. It is hoped that basic issues in olfactory sensory physiology can be explored with this system.

Major Findings:

Last year I reported that we had successfully cultured the odorant responsive cells from the rat olfactory epithelium (OE). These 16 clonal cell strains have been further tested in the past year and they can now be said to correspond to the neuroblast (basal cell) or to an immature neuron precursor. There is much increased interest in cultured cell strains from the olfactory system - both from the neurobiologists and from the biochemists as well. The problem is not unlike that which faced the immunologists some 25 years ago: it was relatively easy to investigate the pool of specificities but very difficult to resolve the single cell specificit in the system. We ultimately want to know what sort of receptors are present on individual cells or clones of individual "blast" cells that differentiate into the sensory neurons. A first approach to this goal could be made by contrasting two or more different lineages of these cells for the physiological responses and for the biochemical differences (eg. 2D gels examined for differnt minor spots etc.) Before this goal could be pursued actively we had to vet the system, i.e. show that the cells we are growing are truly olfactory sensory cells or that they are truly useful models of them. The past year has been spent characterizing our cultures in a way that we now are assured will be satisfactory to the community of researchers in the field.

Because our actively growing cultures lacked obviously convincing markers for the cell type (Olfactory Marker Protein, OMP, carnosine, or diagrammatic morphology) we were led to investigate odorant responses which our immature cells in culture might have. Recent biochemical work with whole OE had established that odorant dependent adenylylcyclase activation was a prominent feature of the system. We have drawn on our experience with endocrine (thyroid) physiology and chosen to measure odorant dependent increases in cAMP. We have also found that there are odorant dependent releases of intracellular calcium. This is the first time that this important pathway has been observed in the olfactory system. Both of these responses are suggestive but don't establish a receptor -> G protein -> cyclase and a receptor -> IP3 -> Ca^{++} mechanism similar to those much studied in endocrine systems. The very existence of a receptor has yet to be demonstrated in the olfactory epithelium. Our main difficulty has been that odorant responses, although unequivocal when they do occur, have prove to vary erratically with different passages of the same clonal cell line. These cultures are complex, consisting of dividing cells and cells in various stages of differentiation; we don't yet know the conditions of passage history and/or medium compostion that might give consistently responsive cell populations. The question was, however, could we publish with what could be construed by a hostile reviewer as irreproducible results?

In March, two things happened to change the picture. First, we found neuron-specific enolase (NSE) in our cells (confirmed by Western blot). Until this finding we could not easily defend our cells as being surely different from fibroblast or the possibly glia-like supporting cells in the OE. The presence of NSE, however, is strong evidence that we have the right cell type because NSE is apparently restricted to neurons and neuroendocrine cells. Second, on the advice of neurophysiologists we have reexamined the "stepdown"

condition in order to try to get better differentiation in our cell lines. We had known that a brief stepdown from high protein containing medium (brain extracts and serum) to serum-free conditions gave in our cells, as it did with neuroblastoma, better neurite outgrowth. To our surprise, moderately dense cultures of our cells, when stepped down to plain DMEM, survived for periods of 3 weeks or more and the cultures resolved themselves into two populations: a very flat cell and a large number of highly neuron-like cells with extended neurites. The interesting thing about these conditions is that as stepdown time and the neuron-like morphology increased, the specific activity of NSE increased as well.

Taken together these two findings have made our erratic odorant response data acceptable as "developmental plasticity" in an otherwise more convincing system. It is ready for publication.

Use of the long term stepdown condition as a test for differentiation has opened other new and potentially exciting doors for us. Of the twenty clones that we selected two years ago, we had been concentrating on only three. These clones were selected with a cross-section of mnorphologies from the original popululation in mind; we could not know the culture appearance of the sensory cell's neuroblast. Two of the "other cell lines" make classical straps of striated muscle when stepped down, two others make typical cartilage nodules (there could be cartilage in the primary cell preps from the turbinate buds sometimes included). One of the lines that made muscle, 41A-8, was included in tests for NSE, and glial fibrillary acidic protein (GFAP) and it proved positive for both! The neuron-like, odorant responsive lines that we had been concentrating on were GFAP negative (all marker protein assays were confirmed by Western blot analysis). At other times, 41A-8 and 41A cultures have been found to make clusters of multilocular fat cells, striated muscle straps, and either glia and neurons or neuroendocrine cells or perhaps a precursor (NSE and GFAP positivity). This situation is strongly reminiscent of the 5-Aza-cytidine effect with 10 T 1/2 cells and the F9 embryonal carcinoma. We shall have to study this effect further since it may well be telling us something profound about the ground rules for culturing stem cells or blast cells wherever they come from.

Publications:

Shunya U, Green N, Coon H, Triche T, Mims S, Berg M. High NaCl induces stable changes in phenotype and karyotype of renal cells in culture. Am J Physicol 1987;253:C230-42.

Miller L, Sakai RK, Romans P, Gwadz RW, Kantoff P, Coon HG. Stable intergration and expression of a bacterial gene in the mosquito anopheles gambiae. Science 1987;230:779-81.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08950-06 LGN

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immunochemistry genetics and function of protein effector molecules

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

P.I.:	Sandra Smith-Gill	Microbiologist	LGN, NCI
	C. Mainhart	Microbiologist	LGN, NCI
	T. B. Lavoie	Biologist	LGN, NCI
	C. Mallett	American Cancer Society Fel.	LGN, NCI
	P. Hamel	Fellow, NCI of Canada	LGN, NCI
	P. Rousseau	Student Volunteer	LGN, NCI

COOPERATING UNITS *(if any)*
A.B. Hartman, Dept. of Biologics, Walter Reed; D. Davies, LMB, NIADKD; B. Brooks, DCRT, NIH; A. Basten University of Sydney, Sydney Australia; University of Cal. Berkeley; E. Appella, LCB, NCI

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	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.)*

Monoclonal antibodies directed against the model protein antigen, lysozyme c, (HEL) are used as probes to study antibody-protein interactions and structures-function relationships, and to study developmentally regulated antigens in normal and neoplastic development. The X-ray structures of two Fab-HEL complexes for 2 of these antibodies (HH5 and HH10) have been refined, and we are now studying in detail the molecular mechanisms underlying specificity and affinity in protein-protein interactions. Site specific mutants of HH10 are being analyzed to determine the contribution of specific amino acids to antibody binding; we are in the process of cloning the rearranged genes for HH5. The gene for HEL has also been expressed in yeast by collaborators at UC Berkeley, and we are analyzing site-specific mutants to determine the contributions of individual amino acids to affinity, antigenicity, and immunogenicity. This system is currently unique in that the 3 dimensional structures of both complexes are known, and we are able to experimentally manipulate both antigen and antibody. In addition, collaborators at Univ. Sydney have made transgenic mice which express HEL, which they recognize as a "self" protein. When these mice are crossed with transgenic mice expressing HH10, the progeny continue to express HEL, but suppress HH10 expression. These transgenic lines provide an excellent model to investigate mechanisms regulating recognition of self, development of tolerance, and other aspects of immune regulation. The development of specificity for lysozyme from an apparently multispecific available antibody repertoire is currently being examined in detail utilizing large panels of hybridoma antibodies. In addition, monoclonal antibodies have been generated against bacterially expressed mouse c-myc protein; these antibodies will be used to purify and characterize structure-function relationships in the myc protein, applying the principles derived from the model protein studies.

Project Description:

We have continued to place major emphasis on investigating antibodies (mAbs) specific for the protein hen egg white lysoyzme (HEL) as a model system for defining the protein-protein interactions underlying antibody recognition of a protein antigen, and the development of such specificity from an apparently multispecific IgM repertoire. We are now in a unique position of being able to manipulate through site specific mutagenesis both antigen and antibodies of two complexes whose x-ray structures have been determined. These results provide a model for studies utilizing mAbs to probe structure-function relationships of biologically important regulatory proteins, such as the protein product of the oncogene c-myc whose structure and function are less well defined.

1. The molecular basis of antibody specificity for a protein antigen

X-ray crystallographic studies are being performed in collaboration with the laboratory of D. Davies (LMB, NIADDK). In the past year, the X-ray structures of two high-affinity ($K_a \approx 2 \times 10^9$) Fab-HEL complexes have been solved and refined. S. Sheriff has refined the complex of HyHEL-5:HEL and E. Padlan is in the final stages of refinement of the HyHEL-10:HEL complex. While the HH5 structure agreed with and confirmed the serologically predicted epitope, the HH10 structure only partially agrees with serological data. In both complexes, the interfaces between antibody and the epitopes are highly complementary in topography, polarity, and charge. The contact between the antibody-combining site and the HEL epitope is extensive and involves 15-17 residues on each surface, and all 6 CDR's of the antibody. The surface of the HH5 antibody-combining site includes a groove which accommodates 2 Arg side changes of HEL; otherwise the surface of interaction between the two proteins is relatively flat although it curls slightly at the edges. The HEL buried surface for the HH5 and HH10 complexes are 750 A^2 and 774 A^2, respectively, while the respective areas buried on the Fab are 721 A^2 and 746 A^2. The area of the interface surfaces for both these antibodies are greater than that of D1.3 (690 A^2 and 680 A^2 for HEL and Ab) the only other anti-protein antibody for which a high resolution X-ray structure of the complexed Ab is available (Amit et al., Science 233: 747); D1.3 has lower affinity (4.5×10^7; ibid). Whether the apparent correlation between the interface surface area and affinity is significant requires further investigation to determine.

We examined the HEL and Fab structures for indications of any significant conformational changes that may have occurred as a result of their association. The HH5 complex suggests that HEL has undergone small but significant concerted changes in the epitope region, and both complexes provide indirect data for induced fit of the antibodies; X-ray coordinates of the uncomplexed Fabs are necessary to determine whether the Fabs have undergone antigen-induced conformational changes. Currently we are concentrating on preparation and purification of Fabs of these two mAbs, in collaboration with E. Silverton, in order to obtain better crystals of the uncomplexed Fabs.

Two different crystal forms of the HH5 Fab-HEL complex were compared; they reveal a difference in the elbow bend of the Fab of about 7°. These results support the hypothesis that the elbow is flexible rather than playing a role in signalling antibody binding to trigger effector functions.

The X-ray structures indicate that linear peptide sequences of HEL are contained within the epitopes recognized by both antibodies. In collaboration with E. Appella (LCB,NCI) we were unable to demonstrate any binding of either antibody to synthetic peptides based upon those linear sequences, even at very high concentrations of both antibody and peptide. These results suggest that the antibodies are very specific for the conformation found in the intact protein. We are also collaborating with M. Geyson (Commonwealth Serum Laboratories, Australia), E. Getzof and J. Tainer (Scripps Institute) to determine whether small synthetic peptides can be used to map the epitopes; this is a unique opportunity to compare the results of peptide mapping with serological mapping for antibodies whose epitopes are known from X-ray crystallography.

T. Lavoie has cloned and expressed the rearranged genes for HH10 and is currently analyzing the binding characteristics of site specific mutants. One intriguing result is that changing a somatically mutated residue in J_H back to the germline sequence sigificantly lowers the affinity and specificity of the antibody for HEL, even though that amino acid is not a contact residue in the complex.

C. Mallett and A. Hartman (Walter Reed), have determined partial nucleotide sequences of the HH5 H and L chains which suggested unusual joining for both the H and L chains. The unusual V-J join in the light chain appears structurally important for antibody complementarity to HEL, in agreement with previous conclusions based upon chain recombination studies. The joining in H3 is also unusual; however, although the conformation of H3 is also unusual, we were not able to attribute H3 conformation or binding characteristics to the unusual sequence, which may be permissive but not necessarily critical to HEL binding. We are now in the process of cloning the rearranged HH5 H and L chain genes to perform site-specific mutagenesis experiments.

We have recently begun a collaboration with the laboratories of J. Kirsch and A.C. Wilson (Dept. of Biochemistry, UC, Berkeley) who have expressed the HEL gene in yeast and are now producing site specific mutants. These mutants are being designed to answer specific questions suggested by examination of the X-ray structures of the complexes. For example, we are examining the importance of salt links to specificity and affinity. While the HH5 complex contains 3 salt links (involving HEL R45 and R68), the HH10 complex appears not to have any. We have shown that an R45 → K mutation reduces affinity of HH5 by at least 500-fold. In contrast, a D → G mutation in the middle of HH10 epitope reduces affinity of that antibody by only 2-4 fold; examination of 6 different amino acid substitutions at that position suggests that the effect on affinity correlates with volume of the side chain, a hypothesis which is being specifically tested by the generation of another series of mutants.

2. Development of specificity repertoires and regulation of the immune response

P. Rousseau analyzed a panel of over 500 hybridomas from polyclonally activated B-cells from both neonatal and adult spleens and found that approximately 50% of the IgM antibodies were reactive with a variety of self and foreign proteins, and of these the majority of antibodies are polyreactive;

these results suggest the normal preimmune repertoire contains a high frequency of polyreactive antibodies. C. Mallett, in collaboration with J. Srinivasapa (LOM, NIDR), has also found that a significant number of the antibodies are organ-reactive or tissue reactive. We are now collaborating with T. Borsos (LMOB NEI) to determine whether IgM anti-self mAbs will fox complement. C. Mallett is currently analyzing the specificity and V-gene repertoires of panels of hybridomas representing the normal development of the immune response from low affinity primary (IgM) to high affinity secondary (IgG) antibodies.

In collaboration with K. Marcu and R. Clynes (Stony Brook) we have examined the specificity and V_H repertoires expressed by a 74 IgM BALB/c plasmacytomas (PCTs) that were induced by pristane and a retroviral construct (RIM virus) containing c-myc and H-ras. The results indicate that V_H usage reflects the germline frequency, and the specificity repertoire is comparable to that of the blast-derived hybridomas examined by P. Rousseau, with the exception that the RIM tumors have a high frequency of phosphorylcholine-specific antibodies. This contrasts with the specificity repertoire of pristane-induced IgA PCTs, and suggests that the RIM virus is targeting a different (probably very early) population of B-cells than is pristane alone. C. Mainhart is currently monitoring a series of experiments designed to test the hypothesis that bone marrow cells are the target population.

In order to examine regulation of tolerance (recognition of self), specificity, and somatic mutation, A. Basten and C. Goodenough (University of Sydney) have made mice transgenic for HEL and for HH10 engineered into a construct that will express the antibody as IgD and IgM. One line of HEL transgenes expressing high HH10 serum levels was crossed with the HEL tolerant transgene; the progeny continued to express HEL, but no longer expressed serum anti-HEL activity. While there were still spleen cells expressing surface HH10, they expressed high IgD-HH10 but low IgM-HH10, in contrast to the parental HH10 transgenes whose splenic cells were surface IgM positive. These results suggest that HH10 anti-"self" clones were suppressed (clonal anergy) but not eliminated (clonal ablation). These mice now present an excellent model for investigating the molecular and cellular mechanisms underlying the recognition of self and tolerance. These mice will also be extremely valuable in immunochemical studies of the physiological and biochemical properties of IgM antibodies, because we will be able to compare homogeneous IgM and IgG antibodies that have the same Fv. The HH10 gene is currently being engineered into a construct that will allow heavy chain switching in transgenic mice, which will allow investigation of class switches and somatic mutation.

3. The structure and function of c-myc protein

In order to define the functional domains of the myc protein, P. Hamel has isolated various fragments of the protein in order to assess their biological activities. A plasmid was constructed using the pRIT2 vector which would produce a fusion protein of Protein A with myc or one of its fragments. The Protein A portion was used to purify the fusion protein. DNA-binding experiments with these fragments have indicated that the DNA-binding domain is encoded by the latter half of exon 3, as has been previously suggested. Furthermore,

fusion proteins containing this domain appear to be dimers, as has been suggested by S. McKnight. To date, DNA mobility-shift assays have not demonstrated sequence specificity of the fusion protein.

In addition, P. Hamel utilized the fusion protein to immunize BALB/c mice and isolated 10 stable hybridoma lines secreting mAbs which bound bacterially expressed c-myc protein (without the Protein A portion). These lines are currently being characterized, both by our laboratory and by a number of collaborating laboratories. One monoclonal, HyMyc-3, stains the nuclei of quail fibroblasts infected with the virus MC29. We plan to utilize these mAbs, once characterized, to analyze functional domains of the myc protein.

Publications:

Hamel PA, Klein MH, Smith-Gill SJ, Dorrington KJ. The relative nonconvalent association-constant between immunoglobulin H- and L-chains is unrelated to their expression or antigen-binding activity. J Immunol 1987;139:3012-20.

Smith-Gill SJ, Hamel PA, Lavoie TB, Dorrington KJ. Contributions of immunoglobulin heavy and light chains to antibody specificity for lysozyme and two haptens. J Immunol 1987;139:4135-44.

Sheriff S, Padlan EA, Silverton EW, Cohen GH, Smith-Gill SJ, Finzel BC, Davies DR. Three-dimensional structure of an antibody-antigen complex. Proc Natl Acad Sci USA 1987;84:8075-79.

Sheriff S, Silverton E, Padlan E, Cohen G, Smith-Gill S, Finzel B, Davies DR. Antibody-antigen complexes: Three dimensional structure and conformational change. In: Sarma RH, Sarma MH, eds. Structure and Expression, 1988; Volume I: Proteins to Ribosomes. Adenine Press, 1988; 49-53.

Hartman AB, Mallett CP, Srinivasappa J, Prabhakar BS, Notkins AL, Smith-Gill SJ. V_H family usage and binding analyses of polyreactive monoclonal autoantibodies derived from nonimmunized adult BALB/c mice. Curr Top Microbiol Immunol 1988; 137:211-215.

D'Hoostelaere LA, Huppi K, Mock B, Mallett C, Gibson D, Hilgers J, Potter M. The organization of the immunoglobulin kappa locus in mice. Curr Top Microbiol Immunol 1988;137:116-129.

D'Hoostelaere LA, Huppi K, Mock B, Mallett C, Potter M. The immunoglobulin kappa light chain allelic groups among the Igk haplotypes and Igk crossover populations suggest a gene order. J Immunol 1988; in press.

Hartman AB, Mallett CP, Sheriff S, Davies DR, Smith-Gill SJ. Unusual joining in heavy and light chains of an anti-lysozyme antibody. J Immunol; in press.

Potter M, Smith-Gill SJ. Physiology of Immunoglobulins. In: Oppenheim J, Shovach E, eds. Textbook of Immunology. Oxford Press, 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08951-06 LGN

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
	S-W. Chung	Visiting Fellow	LGN, NCI
	A. Saxena	Visiting Fellow	LGN, NCI

COOPERATING UNITS *(if any)*

Dr. Francis W. Ruscetti, Laboratory of Molecular Immunoregulation, FCRF, NCI

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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

B

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

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

Investigations on the acute erythroleukemia-inducing spleen focus-forming virus (SFFV) have concentrated on understanding how this virus alters the growth and differentiation of erythroid cells. Erythroid cells from SFFV-infected mice differ from normal erythroid cells in that they can proliferate and differentiate in the apparent absence of the erythroid hormone erythropoietin (Epo). The unique envelope gene carried by SFFV has been shown to be responsible for the biological effects of the virus. Our recent results indicate that SFFV-infected erythroid cells neither secrete an erythroid growth factor that stimulates their growth in an autocrine-like manner nor make detectable levels of Epo-specific mRNA. In addition, SFFV-infected erythroid cells do not differ from their normal counterparts in the number, affinity or size of their Epo receptors. Thus, SFFV is not altering the hormonal requirements of erythoid cells at the level of Epo or its receptor.

Studies on the genetics of susceptibility to erythroleukemia induced by Friend MuLV are concentrating on a gene previously identified on chromosome 5, at or near the Rmcf locus, that encodes a viral envelope glycoprotein related to that of mink cell focus-inducing viruses. RNA transcripts have been identified in tissues from resistant mice that may encode this envelope glycoprotein and cDNA cloning of this putative resistance gene is in progress.

Major Findings:

I. Apparent Epo-independence of $SFFV_P$-infected erythroid cells is not due to Epo production.

Erythroid cells obtained from the spleens of normal mice require Epo for proliferation, while erythroleukemia cells obtained from the spleens of mice infected with $SFFV_P$ are able to proliferate in the absence of added Epo. In order to determine if the SFFV-infected erythroid cells are making growth factors that allow their proliferation in an autocrine-like manner, we prepared conditioned medium from these cells and tested them in a ^3H-thymidine incorporation assay using spleen cells from phenylhydrazine-treated mice. The later cells fail to proliferate unless Epo is added to the medium. Conditioned medium from the IW32 cell line, which secretes Epo, will substitute for Epo in allowing the erythroid precursors from these mice to proliferate. However, conditioned medium prepared in the same way from spleen cells from $SFFV_P$-infected mice fails to stimulate the proliferation of phenylhydrazine spleen cells, even when concentrated 10-fold. In addition, co-cultivation of spleen cells from phenylhydrazine-treated mice with irradiated spleen cells from $SFFV_P$-infected mice failed to stimulate their growth.

Consistent with the above results, antiserum to Epo, which clearly inhibits the proliferation of spleen cells from phenylhydrazine-treated mice, doe not alter the proliferation of spleen cells from $SFFV_P$-infected mice.

To rule out the possibility that spleen cells from SFFV-infected mice synthesize Epo but the factor is not secreted from the cells in detectable amounts or binds to the receptor internally, we examined these cells for the presence of Epo-specific RNA transcripts by Northern blot hybridization analysis. IW32 cells, which constitutively produce Epo, express high levels of the 2 kb Epo-specific mRNA. In contrast, no Epo-specific mRNA could be detected in the spleen cells from $SFFV_P$-infected mice, even when 10 times as much RNA was used. Ethidium bromide straining revealed that comparable levels of 28S and 18S rRNA was transferred to the nitrocellulose filters.

II. Apparent Epo independence of $SFFV_P$-infected cells is not due to differences in the quantity or quality of Epo receptors.

A binding assay utilizing ^{125}I-Epo was developed and shown to be specific for erythroid cells and inhibited only by unlabeled Epo. Using this assay, we have been able to demonstrate that spleen cells from $SFFV_P$-infected mice are not significantly different from spleen cells from phenylhydrazine-treated mice in their ability to bind Epo. Scatchard analysis indictes that both types of cells have approximately the same number of Epo receptors (240-570/cell) and they are of a single class in terms of affinity for Epo (Kd = 140-810 pM).

Chemical cross-linking of ^{125}I-Epo to its receptor on intact erythroid cells from either phenylhydrazine-treated or $SFFV_P$-infected mice results in the labeling of 2 species of proteins that migrate at molecular weights of 135 Kd and 125 Kd. Both bands are absent if excess unlabeled Epo is included during the cross-linking reaction. Assuming that these bands represent the receptor

cross-linked to one ^{125}I-Epo molecule, apparent molecular weights of the receptor molecules would be 100 Kd and 90 Kd. These results are in agreement with those previously reported for spleen cells from $SFFV_A$-infected mice or for normal rat progenitor cells.

III. Sequences responsible for the biological differences between variants of SFFV can be localized to a 120 bp region at the 3' end of the envelope gene.

Two distinct strains of the spleen focus-forming virus, $SFFV_P$ and $SFFV_A$, induce a rapid erythroleukemia in susceptible mice. However, $SFFV_P$-infected cells can proliferate and differentiate without an exogenous supply of erythropoietin, while the $SFFV_A$-infected cells still require a small amount of Epo for differentiation and their proliferation is greatly enhanced by the hormone. The two viruses also differ in the post-translational modification of the product of their envelope genes. Earlier studies have shown that the biological and biochemical differences between these two viruses can be localized to the 3' end of the envelope gene. In order to further dissect this region, we have divided it into two fragments using the Fok I restriction site and have prepared recombinant viruses that contain either the 558 bp Eco RI-Fok I env gene region or the 120 bp Fok I- Cla I env gene region from $SFFV_P$ on an $SFFV_A$ background. Our results indicate that the recombinant virus that contains the 120 bp env gene fragment from $SFFV_P$ retains all of the biological effects of $SFFV_P$ while the recombinant virus that contains the 558 bp env fragment from $SFFV_P$ continues to behave like $SFFV_A$. Since the 120 bp fragment encodes the entire p15E-related hydrophobic transmembrane region of the envelope protein, this suggests that minor differences in the membrane anchoring portion of the protein might be critical in determining Epo responsiveness. We are currently trying to increase the level of expression of these recombinant viruses in order to study the post-translational modification of the envelope glycoproteins, which occurs in only a minor fraction of the total protein pool, in order to determine if the level of expression of the protein at the cell surface also correlates with the biological differences obtained.

IV. Mice carrying the $Rmcf^s$ locus differ from those carrying $Rmcf^r$ in the level of expression of MCF virus-related env gene transcripts.

Previous studies utilizing a series of BALB/c mice congenic for various DBA/2 genes have shown 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. One can detect in these mice a 80K protein related to the env gene of MCF viruses that is thought either to be encoded for by this locus or whose expression is influenced by the locus. Mice carrying $Rmcf^s$ do not express detectable levels of the 80K MCF virus-related envelope glycoprotein. In order to determine if differences in the expression of the MCF-related env gene between mice carrying the 2 loci is also present at the m-RNA level, Northern blot hybridization analyses were carried out using various env gene and LTR probes. Using a synthetic 16 bp MCF env-specific oligomer as a radiolabeled probe, we were able to detect MCF-specific transcripts of 7.2, 6, 3 and 1.8 kb in the spleen, liver and kidney of $Rmcf^r$ congenic mice. The 3 kb and 1.8 kb transcripts were highest in the liver, whereas the 7.2 and 6 kb transcripts were highest in the liver and kidney tissues. Low levels of the 3 and 1.8 kb

transcripts were also seen in all tissues from BALB/c ($Rmcf^s$) mice. No genomic size (8.4 kb) transcripts corresponding to endogenous MCF virus-related proviruses were detected in any of the tissues or strains examined. All of the MCF-related transcripts also hybridized with an LTR probe that is specific for endogenous MCF env-related proviral LTRs. Since the 7.2 and 6 kb transcripts are present in tissues from the $Rmcf^r$ but not the $Rmcf^s$ mouse, either may incode the 80K envelope glycoprotein associated with the $Rmcf^r$ locus. However, it is also possible that the 3 kb transcript, which is expressed at a much higher level in tissues from $Rmcf^r$ mice, may encode the 80K envelope glycoprotein and the $Rmcf^r$ gene is a regulatory gene controlling the level of expression of this transcript.

We are currently attempting to clone the gene encoding the 80K protein expressed in the $Rmcf^r$ mouse using cDNA prepared from liver tissue of this mouse.

Publications:

Frederickson TN, O'Neill RR, Rutledge RA, Theodore TS, Martin MA, Ruscetti SK, Austin JB, Hartley JW. Biologic and molecular characterization of two newly isolated ras-containing murine leukemia viruses. J Virol 1987;61:2109-19.

Jones KS, Ruscetti S, Lilly F. Loss of pathogenicity of spleen focus-forming virus after pseudotyping with Akv. J Virol 1988;62:511-8.

Chung SW, Ruscetti S, Wong PMC. Formation of factor-independent hematopoietic multilineage colonies after Abelson virus infection. Blood 1988;71:973-7.

Wolff L, Chung SW, Ruscetti S. Molecular basis for the pathogenicity of the Friend spleen focus-forming virus. In: Modern Trends in Virology. Springer-Verlag, in press.

Wolff L, Ruscetti S. The spleen focus-forming virus envelope gene, when introduced into mice in the absence of other SFFV genes, induces an acute erythroleukemia. J Virol, in press.

Keller JR, Mantel C, Sing GK, Ellingsworth LR, Ruscetti SK, Ruscetti FW: Transforming growth factor B1 selectively regulates early hematopoietic progenitors and inhibits the growth of IL-3 dependent myeloid leukemia cell lines. J Exp Med, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08952-02 LGN

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Retrovirus-induced myeloid leukemias

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

P.I.:	L. Wolff	Senior Staff Fellow	LGN, NCI
	L. Pasamontes	Visiting Fellow	LGN, NCI
	K. Nason-Burchenal	Graduate Student	LGN, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.75	2.0	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.)*

The overall emphasis of this laboratory is the study of murine myeloid leukemias. Our group has found a useful model in mice for studying these types of leukemias which should help us to determine 1) progressive steps of myeloid tumor development in vivo and 2) some of the aberrantly expressed oncogenes that can contribute to the transformation of such cells. It is hoped that these studies will provide information which is relevant to the understanding of leukemogenesis in man since the two in vivo derived murine tumors described below have similar morphological and functional properties to tumors commonly occurring in man.

Our previous studies had demonstrated that the inflammatory granuloma caused by the injection of pristane into the peritoneal cavity of mice provides a useful system for rapid induction of myeloid tumors by retroviruses. Two retroviral induced tumors that arose in the peritoneal cavity of primed mice were (a) the McML, mature monocyte-macrophage tumors induced by retroviral constructs containing exons 2 and 3 of c-myc cDNA and (b) the MML, promonocytic tumors induced by Moloney murine leukemia virus (M-MuLV) infection and its integration into the c-myb locus. It is not known precisely what makes the granulomatous tissue essential, but it is likely that the inflammatory response recruits potential target cells for transformation from both within and without the peritoneal cavity and produces an environment that is rich in factors that promote cell growth. We have begun to make progress in our attempts 1) to understand the progressive steps in the development of one of the tumors, 2) to determine what is unique about pristane primed mice that allows these diseases to occur and 3) to find a way of inhibiting induction and/or development of the tumors.

Major Findings:

I. Detailed comparison of the biological properties of McML and MML tumors.

Initially it was observed that the c-myc virus induced tumors (McML), and the M-MuLV induced tumors (MML) were quite different morphologically regardless of the fact that they had similar antigenic markers such as Mac-1, Mac-2 and Fc receptors. For example, the McML cells had a mature phenotype in that they had a high cytoplasmic-to-nuclear ratio, numerous cytoplasmic vacuoles and inclusions and ruffled cellular membranes, whereas the MML cells appeared immature having small cytoplasmic-to-nuclear ratios, smooth membranes and often kidney shaped nuclei. The fact that MML cells had weak expression of the Ia antigen on the cell surface compared to the McML also indicated that they were more immature.

In an attempt to characterize the cells more fully, they were examined for a number of phenotypic and functional characteristics typical of myeloid cells. The results of this analysis showed that both tumor types were negative for myeloperoxidase staining but positive for non-specific esterase staining, lysozyme production and phagocytosis, indicating that both tumor cell types are committed to the monocytic series.

Recently it was discovered that responses to growth stimulatory and inhibitory substances were found to vary from one tumor type to another. The McML cells require no adaptation to factor-independent growth in tissue culture, whereas the MML cells adapt slowly, requiring a lag phase of limited growth prior to rapid proliferation. The latter cells, however, can be adapted more quickly in the presence of recombinant granulocyte-macrophage colony stimulating factor (GM-CSF), and one cell line (WIIB 4-9) that has been grown continuously in its presence is now essentially GM-CSF dependent. An additional difference that has been observed between the two in vitro tumor cell lines is their response to the growth inhibitory effects of transforming growth factor-beta (TGF-β). The proliferation of MML cells is completely inhibited whereas proliferation of McML cells is only slightly affected. This difference as well as others mentioned above suggest that the tumors differ in their degree of maturation along the monocytic pathway.

Based on the criteria utilized in our studies so far, the MML cells have been designated promonocytic and appear to be analogous to human acute myelogenous leukemia cells of the M5 subtype, whereas the McML cells are being classified as fully mature monocyte-macrophage cells.

II. Role of the pristane-induced inflammatory response in induction of the viral induced myeloid leukemias.

A. Hematopoietic makeup of the pristane primed mouse. Because of the fact that we have only observed the induction of McML and MML tumors in the pristane primed mouse, studies have been initiated to determine what unique hematopoietic characteristics of primed mice might account for their unique susceptibility. Preliminary studies using an in vitro clonogenic assay have

shown that the spleens of the primed mice have an increased number of precursor cells which are committed to the myeloid lineage; there is at least a 4-fold increase of recombinant GM-CSF responsive cells (CFU-C) relative to the total number of cells in the spleen of the primed mouse when compared to the number of CFU-Cs in the spleen of untreated mice. It is possible that in the primed mouse the spleen becomes an extramedullary site of myelopoiesis providing an increased availability of target cells capable of being transformed by the viruses. We are going to test this possibility in future experiments because there has been evidence that cells capable of being transformed by M-MuLV to become MML are located in a tissue(s) outside of the peritoneal cavity, i.e., intravenously injected, and not intraperitoneally injected mice develop disease.

Consistent with the fact that both tumor types develop ultimately in the spleen is our finding that ascites fluid from pristane primed mice is extremely rich in factors that will support the growth of myeloid cells in vitro.

B. Inhibition of myeloid tumor induction by indomethacin. The effects on tumor induction following treatment of primed mice with indomethacin, a non-steroid antiinflammatory drug, were examined and the results were intriguing. Developing McML and MML tumors responded in dramatically different ways to treatment, in spite of the fact that they belong to the same hematopoietic lineage. In the case of the McML tumors no inhibition was observed during administration of indomethacin in the drinking water, whereas, in the case of the promonocytic MML neoplasms, 100% inhibition was observed.

III. Moloney MuLV-induced myeloid leukemias in different strains of mice.

In order to see if susceptibility to induction of MML by M-MuLV following pristane priming is restricted to BALB/c mice other strains of mice were subjected to the same induction protocol. BALB/cJax and DBA/2 mice, which are resistant to plasmacytoma development after pristane-priming (Potter, Cancer Surveys 3: 247, 1984), were found to be as susceptible to MML induction as BALB/c mice, but C57BL/6 mice were essentially resistant, with only one very late mature macrophage tumor at nearly 6 months post viral infection.

IV. Studies on the induction of myeloid leukemia by viruses closely related to Moloney MuLV.

When some other ecotropic mouse leukemia viruses which are very closely related to Moloney MuLV were inoculated into pristane-primed BALB/c mice it was found that they were unable to induce MML tumors. Retroviruses that were unable to cause disease in this system were the Friend MuLV, a naturally occurring, highly homologous virus, and Mo-MuLV^{sup}, Moloney MuLV which was modified by Jaenisch and colleagues (PNAS 82:1131-1145, 1985) by insertion of the sup^{F} gene into the long terminal repeat region. These studies suggest that Moloney-MuLV contains regions that are unique and very specific for induction of MML tumors.

Publications:

Shen-Ong GLC, Wolff L. Moloney murine leukemia virus-induced myeloid tumors in adult BALB/c mice: requirement of c-myb activation but lack of v-abl involvement. J Virol 1987;61:3721.

Wolff L, Ruscetti S. The spleen focus-forming virus (SFFV) envelope gene, when introduced into mice in the absence of other SFFV genes, induces acute erythroleukemia. J Virol 1988;62:2158.

Wolff L, Mushinski JF, Shen-Ong GLC, Morse HC III. A chronic inflammatory response: its role in supporting the development of c-myc and c-myb related promonocytic and monocytic tumors in BALB/c mice. J Immunol 1988; in press.

SUMMARY STATEMENT

ANNUAL REPORT
LABORATORY OF CELLULAR ONCOLOGY
DCBD, NCI
October 1, 1987 through September 30, 1988

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 analyze 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, the EGF receptor, and papillomaviruses.

We have previously identified a p21-ras effector region (amino acids 31-42) that apparently interacts with the putative ras target(s) and is highly conserved in all ras proteins. In collaborative studies, we have now developed and characterized viral ras genes encoding specific single amino acid substitutions in this region. Mutants with wild type transforming activity, reduced activity, and defective in transforming activity have been derived. Genetic mapping of the region with which GAP (for GTPase activating protein), a cellular protein which can accelerate the intrinsic GTPase activity of a ras proto-oncogene encoded protein but not a highly transforming mutated version of ras indicated that it interacts with this region. The results suggest that GAP may be a p21-ras target.

To demonstrate the oncogenic potential of a full length EGF-receptor (in collaboration with Dr. Pastan's laboratory and Dr. L. Beguinot at the University Microbiology Institute, Copenhagen), an EGFR cDNA from A431 cells was placed in a retrovirus vector which was developed in the LCO. NIH 3T3 cells transfected with the viral DNA or infected with the corresponding rescued retrovirus developed a fully transformed phenotype in vitro that required both functional EGFR expression and the presence of EGF in the growth medium. The results demonstrate that the EGFR virus can efficiently transfer and express EGFR, EGF can be a competence and progression factor for cells with high numbers of EGF-receptors, and increased numbers of EGF-receptors can contribute to the transformed phenotype.

In papillomavirus studies, we have shown previously that the E2 open reading frame, which encodes a product that can trans-activate viral transcription, can bind to a specific motif present several times in bovine papillomavirus (BPV) and other PVs. We have now found this motif, when present in more than one copy, is an E2 dependent enhancer. Using anti-E2 sera, we have detected three E2 protein products in BPV transformed cells. The smaller

forms of E2, which may competitively inhibit enhancement by the full-length E2 product, contain the DNA binding activity but lack enhancer activity. In HPV studies, E7 has been identified as the major transforming gene for NIH 3T3 cells. These results support the hypothosis that E7 may contribute to the transformed phenotype in human tumors.

Biology of fps/fes oncogenes

This project aims at elucidating the biological functions of the normal human c-fps/fes proto-oncogene and understanding the molecular basis of its oncogenic activity. To determine if human c-fps/fes has transforming potential, we have generated a cDNA copy of human c-fps/fes from a genomic DNA by means of a retroviral shuttle vector. The rescued cDNA encoded an NCP92 protein that was indistinguishable from myeloid cell NCP92, providing direct evidence that this 92 Kda cellular tyrosine kinase is the gene product of human c-fps/fes. When the c-fps/fes cDNA was cloned into an efficient retroviral vector that allows the expression of very high levels of the c-fps/fes product NCP92, we have demonstrated for the first time that normal NCP92, when expressed at sufficiently high levels, can induce focal transformation of NIH 3T3 cells. By cloning c-fps/fes into different retroviral vectors, we showed that transforming activity was a direct function of the level of expression of NCP92. The transforming activity of NCP92 could be manipulated at a second level, by modulating its tyrosine kinase activity in vivo. This was done by treatment of infected cells with sodium vanadate, a known inhibitor of tyrosine phosphatases. Vanadate treatment increased the transforming activity of NCP92 by 100-fold. The potentiating effect of vanadate was reversible, dose-dependent, and specific for the c-fps/fes proto-oncogene. We also determined that human c-fps/fes is susceptible to oncogenic activation by N-terminal linkage with viral gag sequences. By several criteria, the transforming activity of gag-c-fps/fes was lower than the transforming activity of gag-v-fps/fes, suggesting that perhaps additional mutations are required to bring out the full transforming potential of human c-fps/fes.

Retroviral replication and cellular oncogene expressions

We have isolated two new strains of human retrovirus. Preliminary results suggest that they may comprise a new type of HIV-like virus. One strain (MCP-1) was isolated from lymph nodes of a patient with mycosis fungoides who was admitted to the Medical College of Pennsylvania. Another strain (HIV_T) was isolated from the blood of a homosexual AIDS patient who came from Malaysia to Taiwan. These viruses exhibited biological characteristics similar to HIV, but several of their properties differed from HIV-1 and HIV-2. These included the ability to superinfect cells that were chronically infected with HIV-1, hybridization with HIV-1 probes only under non-stringent conditions, and distinct divalent cation preference of their reverse transcriptase. These viruses, as well as prototype HIV-1 and SIV, were also found to replicate and show cytopathic effects on some human B and monocytic cell lines as well as on human T cells.

Oncogene studies have involved c-Ki-ras and v-mos. A human embryonal carcinoma cell line was found to be induced to differentiate into neuron-like cells by various treatments including berberine, an alkaloid from a Chinese herbal medicine. This cellular differentiation was accompanied by a down-regulation of expression of the amplified c-Ki-ras2 proto-oncogene. Further

studies on v-mos amplification and expression in helper virus-infected S+L-mink cells revealed that the v-mos protein ($p37^{mos}$), which was abundant in the cells with an anchorage-independent, suspension culture phenotype, was scarcely produced in the ouabain-selected, anchorage-dependent revertant, despite an ample amount of v-mos mRNA. Thus, mechanisms such as instability, or post-transcriptional modulation, of v-mos mRNA are postulated to explain the observation in revertant cells.

Genetic mechanism of neoplastic transformation

We have continued studies on 3.1 kb genomic clone that contains a potential human oncogene originally isolated by us from an African hepatocellular carcinoma cell line. Using this sequence as probe, we have isolated four other genomic clones of related DNA sequences from Chinese and Korean hepatomas; the cell-transformation capability of each on NIH3T3 cells had been ascertained. Transformation of Buffalo rat liver cell (BRL-1) has been achieved by transfection with the original DNA, subcloned in an expression vector carrying a neomycin resistance (Neo^r) marker and a SV40 promoter. Transformed BRL-1 cells, selected by colony-formation in soft-agar, were highly tumorigenic in athymic NIH Swiss nu/nu mice. A diverse tumorigenesis was observed with the transformed NIH3T3 cells in that both solid tumors (75%) at the site of inoculation and B-cell lymphomas (40%) were induced with some challenged mice developing both tumors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03663-12 LCO

PERIOD COVERED
October 1, 1987 through September 30, 1988

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:	D. R. Lowy	Chief, Lab of Cellular Oncology	NCI
OTHER:	J. T. Schiller	Senior Staff Fellow	LCO NCI
	W. S. Sawchuk	Medical Staff Fellow	LCO NCI
	T. J. Velu	Visiting Associate	LCO NCI
	P. Hawley-Nelson	Biotechnology Fellow	LCO NCI
	N. L. Hubbert	Microbiologist	LCO NCI
	A. G. Papageorge	Microbiologist	LCO NCI
	M. A. Pritting	Chemist	LCO NCI

COOPERATING UNITS *(if any)*
See next page.

LAB/BRANCH
Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
9.00	5.00	4.00

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.)*

This program studies oncogenes and papillomaviruses.

Oncogene studies have involved ras and EGFR. To identify cellular ras targets, we have made mutants with changed phenotype in a previously determined effector domain of p21-ras protein and mapped the region of p21-ras with which GAP (GTPase activating protein), which is a cellular protein, interacts to this effector domain. The results suggest that GAP may be a target of p21-ras. Experiments with a full-length EGF receptor proto-oncogene placed in a retrovirus vector have shown that increased numbers of EGF receptors can contribute to the transformed phenotype. EGF can serve as a competence and progression factor in cells with high numbers of EGF receptors.

In papillomavirus studies, BPV E2 protein has been shown to bind to a specific motif present several times in BPV and other PVs. This motif is an enhancer whose activity depends upon E2. Enhancement requires two or more copies of the motif. Anti-E2 sera have detected three E2 protein products in BPV transformed cells. The smaller forms of E2, which may competitively inhibit enhancement by the full-length E2 product, contain the DNA binding activity but lacks enhancer activity. The E7 ORF of HPV-16 is the principal viral transforming gene for NIH 3T3 cells. These results support the hypothosis that E7 can contribute to the transformed phenotype in human tumors.

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

Other Professional Personnel:

W. C. Vass	Biologist	LCO NCI

Cooperating Units:

LMB, NCI, Drs. I. Pastan, G. Merlino, M. Willingham.
LBP, BRMP, NCI, FCRF, Dr. H.-F. Kung.
Tufts New England Medical Center, Boston, Massachusetts, Dr. E. J. Androphy.
Jackson Laboratory, Bar Harbor, Maine, Dr. J. Stone.
University of Iowa School of Medicine, Iowa City, Iowa, Dr. L. Turek.
Hopital Cochin, Paris, France, Dr. P. Tambourin.
University Microbiology Institute, Copenhagen, Denmark, Drs. B. Willumsen and L. Beguinot.
Hebrew University of Jerusalem, Jerusalem, Israel, Dr. A. Levitzki.
University of Erlangen, West Germany, Dr. H. Pfister.
University of Nice, Nice, France, Dr. F. Cuzin.
Cetus Corporation, Emeryville, CA, Dr. F. McCormick

Major findings:

1. ras oncogenes. The principal model system employed to study ras has been the Harvey murine sarcoma virus (Ha-MuSV), whose p21 ras transforming protein differs from its normal cellular proto-oncogene at only 2 amino acids. A major problem of ras biology is to determine how the protein is stimulated to become biologically active and to identify the target(s) with which ras proteins interact. In collaboration with Dr. B. Willumsen, we have identified an effector region (amino acids 31-42) that apparently interacts with the putative ras target(s). This region, which is highly conserved in all ras proteins, is identical in proto-oncogene encoded ras proteins and in highly oncogenic versions of ras, leading us to hypothesize that the viral oncogene and proto-oncogene forms interact with the same target(s). To study this region further and obtain mutants that may help lead to identification of the ras target(s), we have developed and characterized viral ras genes encoding specific single amino acid substitutions in this region (in collaboration with Drs. J. Stone and B. Willumsen). Mutants with wild type transforming activity, reduced activity, and defective in transforming activity have been derived. In collaboration with Dr. F. McCormick and B. Willumsen, we have tested the effect on c-rasH of a newly described cytoplasmic protein called GAP (for GTPase activating protein), which was reported to accelerate the intrinsic GTPase activity of the rasN proto-oncogene encoded protein but not a highly transforming mutated version of rasN. We found that normal and highly transforming rasH encoded proteins function as did rasN. To map the region with which GAP and rasH interact, a large number of deletion and substitution mutants of rasH were analyzed for the ability of GAP to accelerate their GTPase activity. Transformation defective mutants whose lesions were in the effector domain were negative for GAP mediated acceleration; other mutants tested were positive. These results indicate that GAP interacts with the effector domain and suggest that GAP may be a p21-ras target.

2. EGF receptors. A potential role for the normal EGF-receptor gene (EGFR) in tumor formation has been proposed by others from the observation that increased numbers of apparently normal EGF-receptors may be present in various human tumors and tumor cell lines, including the epidermoid carcinoma A431 cell line. To demonstrate the oncogenic potential of a full length EGF-receptor, an EGFR cDNA from A431 cells was placed in a retrovirus vector (in collaboration with Dr. Pastan's laboratory and Dr. L. Beguinot). The vector, which is derived from Ha-MuSV, was developed in the LCO. NIH 3T3 cells transfected with the viral DNA or infected with the corresponding rescued retrovirus developed a fully transformed phenotype in vitro that required both functional EGFR expression and the presence of EGF in the growth medium. The EGFR virus had a high titer (greater than 10^7 focus forming units per ml). Cells expressing about 400,000 EGF-receptors formed tumors in nude mice (which contain endogenous EGF), while control cells did not. Tumors formed even faster in mice that were given exogenous EGF. These results demonstrate that the EGFR virus can efficiently transfer and express EGFR and that increased numbers of EGF-receptors can contribute to the transformed phenotype. The EGFR virus has been used to develop a highly sensitive bioassay that is specific for EGF-receptor ligands. In addition, we have shown that short term EGF treatment of quiescent NIH 3T3 cells carrying high numbers of EGF-receptors is sufficient to simulate DNA synthesis, which implies that EGF can be both a competence and progression factor for cells that overexpress this proto-oncogene.

3. Papillomaviruses. Previous studies have indicated that the bovine papillomavirus (BPV) E2 open reading frame (ORF) encodes a trans-acting factor that can stimulate transcription of BPV sequences located near the viral upstream regulatory region. This ORF now appears to regulate viral transcription positively and negatively. In collaboration with Dr. E. Androphy, we have determined that the ACCGNNNNCGGT motif to which E2 protein binds is an enhancer whose activity depends upon E2. Introduction of the unlinked motif to cells competitively inhibited E2 mediated enhancement. Synthetic oligonucleotides containing this motif functioned as an E2 dependent enhancer element, provided that two or more copies of the motif were present. Single copies of the motif lacked detectable enhancer activity. The DNA binding region of the E2 ORF has been localized to the C-terminal 110 amino acids of the ORF. Anti-E2 sera have detected three E2 encoded proteins in BPV transformed cells; all three are primary translation products of E2. The larger one is the full length (45-kd) protein, while the two smaller ones (28 and 31-kd) are truncated at the N-terminus. All three proteins contain the DNA binding activity, but the smaller form lacks enhancer activity (in collaboration with Dr. L. Turek). The truncated forms of E2 may therefore competitively inhibit enhancement by the full length E2 product.

As part of our studies of papillomavirus transformation, we have studied the ability of HPV-16, the type most commonly associated with cervical carcinoma, to induce cellular transformation of NIH 3T3 cells. In contrast to BPV, whose principal transforming gene on NIH 3T3 cells is E5, we found that E7 is the major transforming gene for HPV-16. This result correlates with E7 (along with E6) being selectively retained and expressed in HPV associated cervical carcinomas.

Publications:

Androphy EJ, Schiller JT, Lowy DR. Analysis of the sequence-specific DNA binding activity of the bovine papillomavirus E2 trans-activating protein. In: Steinberg BM, Brandsma JL, Taichman LB eds. Cancer Cells, Volume 5: Papillomaviruses. New York: Cold Spring Harbor Laboratory, 1987;79-85.

Lowy DR. Potential oncogenic hazards posed by oncogene encoded proteins. In: Petricciani JC, Hennessen W, eds. Developments in Biological Standardization Vol. 68, Background papers from the WHO Study Group on Biologicals, Geneva, 18-19 Nov. 1986. Switzerland: S. Karger, 1987;68:63-7.

Yuspa SH, Lichti U, Strickland J, Jaken S, Lowy DR, Harper J, Roop D, Hennings H. Aberrant regulation of differentiation in epidermal carcinogenesis. In: Colburn NH, Moses HL, Stanbridge EJ, eds. Growth Factors, Tumor Promoters and Cancer Genes, UCLA Symposium, April, 1986. New York: Alan R. Liss, Inc., 1987;183-90.

Velu TJ, Beguinot L, Vass WC, Willingham MC, Merlino GT, Pastan I, Lowy DR. Epidermal growth factor-dependent transformation by a human EGF receptor proto-oncogene, Science 1987;238:1408-10.

Lowy DR, Androphy EJ. Warts. In: Greer KE, ed. Common Problems in Dermatology. Chicago: Year Book Medical Publishers, 1988;431-7.

Lowy DR, Papageorge AG, Vass WC, Willumsen BM. A mutational analysis of ras processing and function. In: Minna JD, Kuehl WM, eds. UCLA Symposia on Molecular and Cellular Biology: Cellular & Molecular Biology of Tumors & Potential Clinical Applications. New York: Alan R. Liss, 1988;203-12.

Ansel JC, Luger TA, Lowy DR, Perry P, Roop DR, Mountz JD. The expression and modulation of IL-1a in murine keratinocytes. J Immunol 1988;140:2274-8.

Hawley-Nelson P, Androphy EJ, Lowy DR, Schiller JT. The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2-dependent enhancer. EMBO J 1988;7:525-31.

Adari H, Lowy DR, Willumsen BW, Der CJ, McCormick F. Guanosine triphosphatase activating protein (GAP) interacts with the p21 ras effector binding domain. Science 1988;240:518-21.

Stone JC, Vass WC, Willumsen BM, Lowy DR. p21-ras effector domain mutants constructed by "cassette" mutagenesis. Mol Cell Biol 1988. In press.

Hubbert NL, Schiller JT, Lowy DR, Androphy EJ. Bovine papillomavirus Transformed cells contain multiple E2 proteins. Proc Natl Acad Sci USA 1988. In press.

Vousden KH, Doniger J, DiPaolo JA, Lowy DR. The E7 open reading frame of human papillomavirus type 16 encodes a transforming gene. Oncogene Res 1988. In press.

Pandiella A, Beguinot L, Velu TJ, Meldolesi. Transmembrane signaling at EGF receptors everexpressed in NIH-3T3 cells: phosphoinositide hydrolysis, cytosolic Ca-2+ increase and alkalinization correlate with EGF-induced cell proliferation. Eur J Biochem 1988. In press.

Velu TJ, Beguinot L, Vass WC, Zhang K, Pastan I, Lowy DR. Retroviruses expressing different levels of the normal epidermal growth factor receptor: biological properties and new bioassay. J Cell Biochem 1988. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09051-03 LCO

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Biology of fps/fes oncogenes

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

PI: R. A. Feldman Senior Staff Fellow LCO NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

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.)*

This project aims to elucidate the biological function(s) of the human c-fps/fes proto-oncogene and to understand the molecular basis of its oncogenic potential. The coding sequence of human c-fps/fes was cloned into an efficient retroviral vector that allows the expression of very high levels of the c-fps/fes product NCP92. Using this DNA or rescued infectious virus, we demonstrated for the first time that when normal NCP92 was expressed at sufficiently high levels, it caused cell transformation in NIH 3T3 cells. By cloning c-fps/fes into different retroviral vectors, we showed that transforming activity was a direct function of the levels of expression of NCP92. The transforming activity of NCP92 could be manipulated at a second level, by modulating its tyrosine kinase activity in vivo. This was done by treatment of infected cells with sodium vanadate, a known inhibitor of tyrosine phosphatases. Vanadate treatment increased the transforming activity of NCP92 by 100-fold. The potentiating effect of vanadate was reversible, dose-dependent, and specific for the c-fps/fes proto-oncogene.

Major Findings:

The gene product of human c-fps/fes is a 92 Kda cellular tyrosine kinase (NCP92) that is specifically expressed in myeloid cells. The pattern of expression of NCP92 in certain leukemic cell lines suggests that this protein may be involved in the cellular response to factors that promote growth and/or differentiation of myeloid cells [Proc. Natl. Acad. Sci. USA 82:2379-2383 (1985)]. To further our understanding of the biology of c-fps/fes, we have cloned a cDNA copy of this gene and have begun the characterization of its biological and biochemical properties.

We have recently shown that when the c-fps/fes cDNA was cloned into a Feline sarcoma virus-derived retroviral vector, NCP92 was expressed in NIH 3T3 transfectants at 5-10 times the levels found in the high producer monoblastic U937 cell line. At these levels of expression, NCP92 did not transform NIH 3T3 cells. However, modification of the N-terminus of NCP92 by fusion with viral gag sequences, activated its transforming potential. Although spontaneous chromosomal rearrangements involving the c-fps/fes locus have not yet been reported, this observation suggests that human c-fps/fes might also be susceptible to oncogenic activation by chromosomal alterations that modify the N-terminus of NCP92 (1).

Since the lack of transforming activity of unmodified NCP92 described above could have been due to insufficient levels of NCP92 expression, we have now re-examined this question by cloning the coding sequence of NCP92 into two higly efficient retroviral vectors. Using these vectors we obtained very high levels of NCP92 expression, and we were able to rescue high titer virus. The first construction, pFF, contained a Friend leukemia virus-derived long terminal repeat, and the second construction, pHF, contained a Harvey sarcoma virus-derived long terminal repeat. After transfection of these DNAs into NIH 3T3 cells and G418 selection, pFF transfectants expressed up to 200 times higher levels of NCP92 than U937 cells, and many of these transfectants were fully transformed. pHF transfectants expressed up to 50 times the levels of NCP92 compared to U937 cells, but in this case the number of tranformants was very low. Upon rescue with amphotropic helper virus, cells transformed with pFF and pHF yielded transforming viruses whose titers were 10^3-10^4 FFU/ml, and 10^1-10^3 FFU/ml, respectively. By end point cloning, virus titers were increased by one to two orders of magnitude. These results demonstrated that overexpression of the c-fps/fes proto-oncogene can cause cell transformation, and that transforming activity was a function of the level of NCP92 expression.

We also showed that the transforming activity of c-fps/fes can be manipulated at a second level, by modulating the enzymatic activity of NCP92 in vivo. For this purpose, we treated cells infected with pFF and pHF with low concentrations of sodium vanadate. Vanadate is a known inhibitor of tyrosine phosphatases, and therefore, it might be expected to prevent the dephosphorylation of NCP92 targets in vivo, and thus potentiate its transforming activity. The incubation of infected cells with 4 uM vanadate increased the apparent titer of transforming virus by two orders of magnitude. This effect was reversible and dose dependent. These results suggest that the kinase activity of NCP92 plays an important role in its mechanism of transformation. Our findings also suggest that there are no tyrosine sites in the NCP92 molecule

whose phosphorylation inhibits NCP92 kinase activity. This is consistent with DNA sequence data which shows that no negative regulation site equivalent to tyrosine 527 in $p60^{c\text{-}src}$, exists in NCP92. On the contrary, phosphorylation of the only known tyrosine phosphorylation site in NCP92 (tyrosine 713), might act as a positive regulator of its kinase activity. Vanadate was able to discriminate between different fps/fes genes according to their transforming activity: its potentiating effect was maximum with c-fps/fes, it was nil with the highly transforming v-fps/fes oncogene, and it was intermediate with gag-c-fps/fes, which was more transforming than c-fps/fes, but less transforming than v-fps/fes.

In contrast to c-fps/fes, vanadate was slightly inhibitory to the transforming activity of other cellular tyrosine kinases, such as $p60^{c\text{-}src}$ and EGF receptor. This result is consistent with the idea that different cellular tyrosine kinases have different modes of action and biochemical properties.

The availability of high titer retroviruses carrying the human c-fps/fes proto-oncogene should be very useful to study its biological function(s) in normal and leukemic myeloid cells, and to understand the molecular basis of its oncogenic potential.

Publications:

Feldman RA, Vass WC, Tambourin PE. Human cellular fps/fes cDNA rescued via retroviral shuttle vector encodes myeloid cell NCP92 and has transforming potential, Oncogene Res 1987;1:441-58.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05550-19 LCO

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Retrovirus replication and cellular oncogene expressions

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

PI:	K. S. S. Chang	Medical Officer	LCO NCI
OTHER:	L.-C. Wang	Visiting Associate	LCO NCI
	C. Gao	Guest Researcher	LCO NCI

COOPERATING UNITS *(if any)*

See next page.

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.00	3.00	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.)*

In studies on two new strains of human retrovirus, preliminary results suggest that these may comprise a new type of HIV-like virus. One strain (MCP-1) was isolated from lymph nodes of a patient with mycosis fungoides who was admitted to the Medical College of Pennsylvania. Another strain (HIV_T) was isolated from the blood of a homosexual AIDS patient who came from Malaysia to Taiwan. These viruses exhibited biological characteristics similar to HIV, but their molecular properties differed from HIV-1 and HIV-2. Further tests for molecular characterization are in progress. These viruses, as well as prototype HIV-1 and SIV, were also found to replicate and show cytopathic effects on some human B and monocytic cell lines as well as on human T cells.

Oncogene studies have involved c-Ki-*ras* and v-*mos*. A human embryonal carcinoma cell line was found to be induced to differentiate into neuron-like cells by various treatments including berberine, an alkaloid from a Chinese herbal medicine. This cellular differentiation is accompanied by a down-regulation of expression of the amplified c-Ki-*ras*2 protooncogene. Further studies on v-*mos* amplification and expression in helper virus-infected S+L-mink cells revealed that the v-*mos* protein ($p37^{mos}$), which was abundant in the cells with an anchorage-independent, suspension culture phenotype, was scarcely produced in the ouabain-selected, anchorage-dependent revertant, despite an ample amount of v-*mos* mRNA. Thus, mechanisms such as instability, or post-transcriptional modulation, of v-*mos* mRNA are postulated to explain the observation in revertant cells. Screening of a cDNA library constructed from mRNA of SJL/J mouse reticulum cell neoplasm (RCN) with various probes resulted in isolation of a number of cDNA clones which are being further tested for their ability to hybridize with mRNAs of RCN and normal mouse cells in a differential manner.

Cooperating Units:

Laboratory of Molecular Oncology, NCI, Dr. A. Seth.
Biotech Research Laboratories, Rockville, Maryland, Dr. S. Alexander.
Center for Disease Control, Atlanta, Georgia, Dr. C. Y. Ou.
New England Regional Primate Center, Harvard Medical School, Dr. Y. Li.
Institute of Biomedical Sciences, Academia Sinica, Taiwan, Dr. W. Liu.

Major Findings:

1. Isolation and characterization of viruses related to HIV (human immunodeficiency virus). A strain of retrovirus (MCP-1) was isolated from lymph nodes of a female patient, age 62, who was treated for mycosis fungoides at the Medical College of Pennsylvania. The virus particles were ca. 100 nm in diameter, budded from cells, and contained envelope and cylindrical nucleoid structures. The virus showed reverse transcriptase activity, and the infected CD4-positive T cells showed cytopathic effects (CPE) with various degrees of syncytial cell formation. Another strain of retrovirus (HIV_T) was isolated from the peripheral blood lymphocytes of an AIDS patient, a young homosexual Chinese male, who came from Malaysia to Taiwan. This virus also exhibited HIV-like characteristics as did MCP-1 virus, but they were different from each other in some biological characteristics, such as host ranges and antigenic properties by Western blot analysis. These viruses showed characteristics different from the prototype HIV-1 ($HTLV\text{-}III_B$): Their reverse transcriptase activities were more efficient with Mn than with Mg ions, whether oligo(dT)-poly(rA) or oligo(dG)-poly(rC) was used as a primer-template; these viruses could superinfect and cause CPE on chronic HIV-releasing T cells, suggesting that these viruses have envelope proteins different from that of the prototype HIV-1 virus, as shown by the lack of viral interference; Western blot analysis revealed only the presence of p24 and gp41 cross-reactive with human HIV-1 antiserum but not with human HIV-2 antiserum; Southern blots of DNAs from cells infected with these viruses showed no hybridization reaction with the probes containing a whole HIV-1 genome (pBH10-R3) but showed a cross-hybridization reaction when probed with a 7.4 kilobase SIV BamHI fragment only at conditions of reduced stringency. These viruses did not infect or produce CPE in fibroblasts of human, rabbit, or mouse origin. Hybridization tests with HTLV-I, II, and V probes were also negative. By polymerase chain reaction using different synthetic oligonucleotides as primers for amplification of a portion of the viral genome, it was demonstrated that no sequence homology existed between these viruses and the prototype HIV-1, except that a portion of gag gene sequence (from nucleotide No. 1551 to 1665) showed a homology. Further work is in progress to molecularly clone these viruses and to determine their relationships with the known viruses.

2. Replication and cytopathic effects of human and simian immunodeficiency viruses in human B and monocytic cell lines. In attempts to find if the lymphocytic cell lines established from patients with diseases caused by genetic abnormalities are more susceptible to replication and cytopathicity of HIV and SIV, we obtained lymphocyte cell lines from xeroderma pigmentosum and Lesch-Nyhan disease patients, and infected these cells with HIV or SIV. Three isolates of each of HIV and SIV were tested. Using monoclonal antibodies against surface markers, and by immunofluorescent flow cytometry analysis,

these cells were characterized as B cells which exhibited Leu12(CD19), LeuM1(CD15), and HLA-DR antigens. In addition, other human CD4-positive T cells, and monocytic cell lines were tested. The viral replication was monitored by reverse transcriptase assay of the culture supernatants, and the CPE was assessed by trypan blue staining. It was found that, not only some human T cells, but also some of these B cells and monocytic cells supported HIV and SIV replication, and showed marked CPE within 7-10 days postinfection. The presence of EB virus genome in the cell is not necessry for expression of CPE, because some cells that lack EB virus genome also showed CPE. The specificity of the CPE was confirmed by its inhibition with neutralizing antibodies. Since these cells express little or no CD4 antigen which is recognized as the virus receptor, other mechanisms of virus entry such as endocytosis (viropexis) may be postulated.

3. Down-regulation of c-Ki-ras2 gene expression in human embryonal carcinoma cells treated with agents to induce differentiation. Despite the presence of amplified c-Ki-ras2 gene and overexpression of its mRNA, the human embryonal carcinoma cell line, Tera2/clone D1, showed a marked reduction in its Ki-ras mRNA production, down to the level of that of normal fibroblasts, after treatment with retinoic acid (10^{-7} M) or berberine (0.1 ug/ml), an alkaloid extracted from a Chinese herbal medicine. When treated with these agents for 4-5 days, the c-Ki-ras2 mRNA began to decline, and the cells began to differentiate into cells morphologically similar to neuronal cells. These cells stop replication and remain attached to the substratum and survive for more than 10-14 days. This system can be further applied to study the epigenetic effect of regulation of oncogenicity.

4. Expression of v-mos protein in helper virus-infected S+L-mink cells. In cooperation with Dr. A. Seth, the amount of v-mos protein was examined for the four different clones of superinfected S+L-mink cells. It was found that only the clone that showed an anchorage-independent, suspension culture (AISC) phenotype exhibited a marked band of $p37^{mos}$ in its Western blot, the specificity of which was confirmed by competitive blocking by the synthetic mos peptide. The clone that reverted to the anchorage-dependent phenotype showed a faint band of $p37^{mos}$ despite the fact that it also produced as much v-mos mRNA as that produced by the clone with AISC phenotype. Therefore, it is possible that the mRNA produced by the revertant is unstable and degraded before translation, or other post-transcriptional modulation may result in marked reduction in production of $p37^{mos}$ protein. These results suggest that the oncogenicity and metastatic potential in nude mice of these clones are well correlated with the amount of $p37^{mos}$ protein rather, than with the amount of v-mos mRNA or extent of v-mos gene amplification in the cellular DNA.

5. Search for oncogene associated with the spontaneous reticulum cell neoplasm (RCN) in SJL/J mice. Previous studies by us revealed that, in the RCN tumor DNA, there were additional HindIII fragments, 9-19 kilobase in size, which hybridized with the v-abl and ecotropic virus LTR probes, and absent in the control normal mouse DNA. A cDNA library was constructed from the poly(A)$^+$RNA of RCN tissues (lymph nodes and spleens). The cDNA was inserted into the lambdaZAP vector which is able to automatically excise the insert region with a helper phage and circularize it, forming a Bluescript plasmid. After screening with the 9-19 kb HindIII fragments, several clones with a single, or multiple

EcoRI inserts were selected. These inserts are being isolated and nick-translated to be used as probes for hybridization with the mRNAs derived from RCN or control, normal mouse tissues. The insert which hybridizes with the RCN mRNA but scarcely with that of normal mouse tissues would be selected for further study. Furthermore, the cDNA library was also screened with v-abl, and LTR as probes. Three clones were obtained with the former, and more than 200 with the latter probe. By screening these inserts for their differential ability to hybridize to RCN versus normal tissue mRNA, and by analysing the biological functions as well as the molecular structure of genes detected by these probes, it would be possible to elucidate the nature of the oncogene associated with development of spontaneous RCN and other pre-B cell tumors.

Publications:

Wang L-C, Vass WC, Gao C, Chang KSS. Amplification and enhanced expression of the c-Ki-ras2 protooncogene in human embryonal carcinomas, Cancer Res 1987; 47:4192-8.

Chang KSS, Wang L-C, Gao C. Variants of amphotropic type-C retrovirus isolated from cultures of Moloney- and Rauscher-MuLV-induced tumors, International J Cancer. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04834-12 LCO

PERIOD COVERED
October 1, 1987 through September 30, 1988

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

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
	M. Farshid	Guest Researcher	LCO NCI

COOPERATING UNITS *(if any)* B.J. Park, Seoul National Univ. Coll. Medicine, Seoul, Korea
P. Cohen, LP, NCI
N. A. Wivel, GCRCP, NIH
E. Butler, LDN, NICHD

LAB/BRANCH
Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.00	1.00	2.00

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 thrust of this study is to elucidate the genetic mechanism of neoplastic transformation of normal tissues. The experimental system centers on a 3.1 kilobasepair (kb), moderately cell-transforming human oncogene, hhc^M, isolated from an African hepatocellular carcinoma cell line, Mahlavu. Using hhc^M as probe, we isolated four other genomic clones of hhc^M-related DNA sequences from Chinese and Korean hepatomas; the cell-transformation capability of each on NIH3T3 cells had been ascertained. Transformation of Buffalo rat liver cell (BRL-1) has been achieved by transfection with hhc^M 3.1 kb DNA, subcloned (rpNpM-1) in an expression vector carrying a neomycin resistance (Neo^r) marker and a SV40 promoter. Transformed BRL-1 cells, selected by colony-formation in soft-agar, were highly tumorigenic in athymic NIH Swiss nu/nu mice. A more diversified tumorigenesis was observed with rpNpM-1 DNA transformed NIH3T3 cells. Both solid tumor (75%) at the site of inoculation and B-cell lymphomas (40%) were induced with some challenged mice developed both tumors.

As an on-going interest the role and relationship of hepatitis B virus (HBV) with hhc^M in hepatocarcinogenesis were analyzed. Results of nucleotide homology search indicated that the human flanking sequence for HBV integration was seen in hhc^M, involving a PstI recognition sequence. Only 4/6 nucleotides of PstI was seen at the same location of the normal liver homologue. HBV sequence was seen at a 2.0 kb site elsewhere in the hepatoma. Several aflatoxin B_1 mutagenized hhc^M clones were recovered from the tumor developed by inoculating athymic nu/nu mice with NIH3T3 cells transfected with AFB_1-bound hhc^M 3.1 kb DNA. These mutagenized hhc^M clones would be analyzed to resolve the specific dG involved in the AFB_1 induced mutation.

Major Findings:

1. The biological effect(s) of hhc^M and related DNA sequences.

In this on-going project studying the molecular biology of the oncogene isolated from human (Mahlavu) hepatocellular carcinoma cells, we isolated several genomic DNA clones from four other Chinese and Korean hepatoma DNAs using a [^{32}P]hhc^M probe and DNA transfection assay on NIH3T3 cells; they are hhc^{CC}, hhc^{K3}, hhc^{K4} and hhc^{K14}. Progress was also made on 1) transformation of liver cells (BRL-1) with hhc^M; 2) Soft agar cloning; and 3) tumorigenesis of the transformed NIH3T3 and BRL-1 cells in athymic Swiss nu/nu mice.

To transform the target Buffalo rat liver cells, BRL-1, we made the following constructs with the original Mahlavu 3.1 kb hhc^M DNA ligated to a neomycin resistance (Neo^r) vector carrying a SV40 promoter and enhancer to provide a marker, Neo^r; they are rpNpM-1, rpNpM-2, and rpNM. Both rpNpM-1 and rpNpM-2 measure 11.6 kb but differ from each other in orientation. rpNM measures 8.9 kb. Transfection experiments with any one of the recombinant DNAs from rpNpM-1, rpNpM-2, rpNM, and Neo^r usually yielded 10^3 colonies ug^{-1} DNA. Although all selected colonies expressed Neo^r initially, dependent on the construct, Neo^r was either inhibited from phenotypic expression or not transmitted stably in the tumor cells. Tumor cells established from rpNM DNA transfected NIH3T3 cells eventually lost the Neo^r characteristic and could not survive G418 selection. Cells transfected with rpNpM-2 DNA retained Neo^r phenotype but showed low efficiency in soft-agar colony formation. Construct rpNpM-1 was most stable; Neo^r phenotype was transmitted through secondary and tertiary transfections. It is especially useful for studying hhc^M expression through the action of an SV40 Promoter.

Transformed BRL-1 cells, like transformed NIH3T3 cells, showed clusters of round and refractile cells resembling a focal transformation; they also showed signs of active cytoplasmic synthesis, such as secretory vacuoles. Southern-blot hybridization analysis on DNA prepared from a single colony, against a [^{32}P]hhc^M probe, confirmed the incorporation of human 3.1 kb DNA in NIH3T3 or BRL-1 cells transfected with rpNpM-1 DNA. Transformed cells formed colonies in soft agar with great efficiency and were highly tumorigenic in athymic Swiss nu/nu mice.

Tumorigenicity of the rpNpM-1 DNA transformed NIH3T3 cells can be summed up in one typical series (out of 5) of 12 mice challenged subcutaneously with 10^5 to 10^6 cells: 6 developed tumors at site of inoculation; one developed both solid tumor and lymphoma; 3 developed lymphomas within 9-15 weeks; one developed ascites and lymphomas; and one died. NIH3T3 cells transformed with rpNpM-2 DNA or rpNM DNA developed only solid tumors. Control NIH3T3 or BRL-1 cells transfected with Neo^r DNA, did not form tumors or lymphomas in athymic Swiss nu/nu mice. BRL-1 cells transformed with rpNpM-1 DNA developed tumors so fast that lymphadenopathy was not evident at sacrifice of the mice.

2. Induction of B-cell lymphoma in athymic nu/nu mice.

The gross pathology of the tumor-bearing mouse challenged with rpNpM-1 DNA transformed NIH3T3 cells, revealed generalized lymphadenopathy and the histo-

pathology indicated that in addition to the involvement of spleen and lymphoid tissues, wide-spread infiltration of lymphocytes into other organs such as brain, liver, kidney and lung occurred; the appearance was consistent with diffuse histiocytic lymphoma. Direct immmunofluorescence assays with specific typing anti-sera on live cells prepared from spleen and various lymph nodes in short-term culture, suggested the cell type as B-cell lymphoma. This provides an experimental model not only for studying possible oncogene activation but also for studying the various interactions involved in signal transduction essential for the activation of B-cell proliferation and differentiation.

The role of hhc^M in the induction of B-cell lymphomas in these challenged mice was examined by comparing the hhc^M sequence with one published DNA sequence of the Bcl gene (Negrini et al., Cell 49:455-463, 1987). No significant homology was observed between the two. hhc^M DNA however hybridized strongly with a chromosome 18 probe, on which dbl was located. The induction of B-cell lymphomas in athymic nu/nu mice by rpNpM-1 DNA transformed cells was probably mediated through a mechanism that has yet to be determined.

3. Relationship between hhc^M and HBV DNA sequences.

Recent epidemiological data implicated HBV infection with the development of hepatocellular carcinoma in the Far East, Taiwan, Southeast Asia and certain areas of Africa. We have completed a survey on the HBV DNA sequence integration in the hepatoma DNAs from 17 Koreans, Mahlavu and 2 Chinese. The available serological data on HBV infection and serum alpha-fetal protein levels provided with the Korean hepatomas were also evaluated. We found that seroconversion in the patient might not be correlated with HBV DNA integration in host DNA. Within the same hepatoma, HBV DNA copy number was usually low (4 copies/diploid genome) but hhc^M level was high (20-48 copies/diploid genome). Taking into consideration the hhc^M sequence, results of this survey suggest that HBV attributes to oncogenesis in the case of Mahlavu, probably via an activation mechanism involving possibly an integration of transient interaction of HBV DNA with hepatocyte DNA, leading to recomination and rearrangement and eventual amplifications of hhc^M DNA sequences.

4. Sequence comparison between hhc^M and c-hhc (putative cellular homolog).

The DNA sequence of the hhc^M 5' terminus was aligned with that of the 5' terminus of c-hhc. Although co-linear alignment was obtained for long stretches of sequence, divergency of hhc^M sequences from c-hhc was seen at sites repeated sequences and inversions in the hhc^M. One site of interest was the Pst I recognition site in the cellular flanking sequence for HBV virus integration present in the hhc^M but not in the c-hhc sequence. In c-hhc, only 4 out of 6 PstI bases were present.

5. Aflatoxin B_1 targets on hhc^M and cloning of the AFB_1 mutagenized hhc^M.

The number of preferential AFB_1 binding dG sites (i.e., the most probable AFB_1 induced point-mutations) on hhc^M was estimated to be about 86, by computer analysis of hhc^M sequence, based on the empirical rules that we formulated from detailed analysis of AFB_1 bound double-stranded DNA which was in agreement with other published data. We have examined the effect of nine of the predicted 86

point-mutation sites (i.e., G.C to T.A transversion) by utilizing synthetic polynucleotides containing this transversion in site-mutagenesis study. None of the nine selected in this study proved to be critical for affecting the cell-transformation activity. We have isolated 3 clones of the mutagenized hhc^M DNA sequence from the DNA of tumor cells of NIH3T3 transformant which was transfected with AFB_1-activated 3.1 kb hhc^M DNA. The restriction map of these clones are currently being verified prior to sequence analysis.

Publications:

Yang SS, Vieira W, Farshid M, Cannon G, Wivel NA. Induction of B-cell Lymphomas in Athymic NIH Swiss nu/nu Mice. Leukemia 1988. In press.

SUMMARY
Annual Report of the Laboratory of Biochemistry, National Cancer Institute
October 1, 1987 to September 30, 1988

The diversity and high quality of research in the Laboratory of Biochemistry reflects the different interests and expertise of the fourteen independent groups which constitute the laboratory. Two members of the laboratory were recognized this year for their outstanding contributions. Dr. Bruce Paterson received the NIH Award of Merit and Dr. Samuel Wilson the Meritorious Service medal. Many of us were invited to give Lectures at National and International Conferences.

The advantages of our arrangement of having many independent investigators leading their own research programs have been evident over the last few years. It brought to the laboratory a broad and complementary array of technical expertise in molecular biology and genetics, protein biochemistry and immunology. It facilitates attracting and keeping in the laboratory first class scientists such as our most recently arrived colleagues, Drs. Wu, Yarmolinsky and Lichten. The common goal of this diverse group of investigators is to solve basic mechanisms of cellular regulation, a theme of crucial importance to the overall goal of the Institute. The convergence of interests is facilitated by close spatial relationships and constant interchange of ideas and provides a most efficient utilization of space and resources. All these advantages were deeply felt by members of the Laboratory when they learned of the departure of Dr. Maxine Singer, chief of the Laboratory for the past seven years, to become President of the Carnegie Institution of Washington. We all miss Maxine's administrative support and guidance but are very glad that she has accepted to continue to lead a research group in the Laboratory and thereby actively participate in its scientific life. The composition and scientific contributions of the laboratory as described below are the result of Maxine's leadership and we hope to continue her tradition .

THE CONTROL OF GENE EXPRESSION.

The regulation of gene expression continues to be the focus of interest of many members of the Laboratory.

Carl Wu leads an active research group studying the regulation of gene expression in Drosophila. His group progressed in the cloning of the gene for the protein activator of heat shock loci. Their purification procedure has yielded sufficient amounts of protein for the preparation of polyclonal antibodies. These antibodies were used to screen a lambda gt11 expression library. A candidate clone has been isolated and is being sequenced. Concurrently peptides resulting from CNBr or proteolytic cleavage of the protein are being subjected to protein microsequencing. This strategy should lead to the identification of the authentic gene and should open the way for a detailed analysis of the structure and function of this transcription factor, the key transducer of the heat shock-induced signal to the transcriptional machinery. The recent identification of the functional role of the heat shock protein has brought additional interest to this problem. This group is also involved in a more general biochemical screen of DNA-binding factors that interact with the Drosophila segmentation gene fushi tarazu. One of the factors has been purified to homogeneity, and purification of a second factor has been initiated.

Bruce Paterson and his colleagues' major efforts are devoted to the study of the regulation of genes associated with the differentiation of muscle. They previously defined the cis-acting elements involved in the regulation of expression of various actin genes. In addition to the cis-elements, located 5' of the α-actin gene, they have identified and sequenced a part of the β-actin gene in the 3' untranslated region which imparts the regulatory pattern of the β-actin gene to any other appropriately placed gene. They are now focusing their efforts on the isolation, from nuclear extracts of myoblasts and myotubes, of proteins that bind to these cis-elements. Different issues are raised in studies of myosin light chain gene expression. A single gene encodes light chains 1 and 3 utilizing two promoters and differential splicing of different sets of exons. The promoter sequences for the light chain gene are essentially identical in rodents and chicken but the chicken promoters are

silent. They have shown that a species-specific enhancer, recently isolated from the rat gene, is responsible for the stimulation of the rodent promoter. 5-bromodeoxyuridine, which blocks myogenesis in muscle cells, has been used by this group to demonstrate the specific suppression of the activators of myogenic genes by this analog, which leaves housekeeping genes fully active. Expression of a specific gene in cells induced to differentiate into neuronal cells by nerve growth factor is also under investigation. The expression of this gene is restricted to neuronal cells. Other studies involve the cloning of myosin genes of Acanthamoeba castellanii and yeast.

The regulation and function of metallothionein continues to serve as a fruitful system for study by Dean Hamer and his colleagues. Working in the simple unicellular eukaryote, S. cerevisae, and in the more complex cells of mouse and man, they have pinpointed the cis-acting metallothionein regulatory sequences to the level of the individual nucleotides. They have also identified several trans-acting regulatory factors, and the gene for one of these has been cloned from yeast. Other studies involve the identification of critical cysteine residues in yeast metallothionein and the role of metallothionein in resistance to the anti-tumor drug cis-platinum.

Dr. Edward Kuff's group has completed a structural analysis of the endogenous mouse intracysternal A particle (IAP)-related transposable retroviral elements and begun to study the regulation of IAP expression at the molecular and biochemical levels. The normal mouse thymus, where levels of IAP expression vary greatly among inbred strains, is being used to analyze the number, identity and genomic environment of active genes. Interactions of the IAP long terminal repeats (LTRs) with nuclear regulatory proteins is being studied in a cell free system containing histone-depleted nuclear extracts and a cloned IAP LTR known to be promoter-competent in vivo. Collaborative studies are being continued on the possible involvement of IAP protein expression in the pathogenesis of a genetically determined autoimmune-mediated diabetes of mice. In the same group, Dr. Kira Lueders has demonstrated that myeloma cells, in which variant IAP elements have been amplified, contain linear proviral forms of the elements. A particle-associated endonuclease activity, detected in these cells, may be encoded by these elements as a result of an unusual splicing to a site introduced into the element by a short insertion.

The major interest of Dr. Beverly Peterkofsky is the regulation of the synthesis of the extracellular matrix components: collagen and proteoglycan. Several years ago Dr. Peterkofsky and her colleagues proposed that ascorbate plays an indirect role in the regulation of synthesis of the components of the extracellular matrix. Dr. Peterkofsky's model implied that scurvy-induced fasting leads to hormonal changes that regulate matrix formation. This hypothesis has now received experimental support: sera from fasted (ascorbate supplemeted), and scorbutic guinea pigs contain decreased levels of insulin-like growth factor I (IGF-I or somatomedin C). Sera from both groups of animals support reduced levels of mitogenesis in 3T3 cells and of extracellular matrix synthesis in cultured chondrocytes. These defects are caused by the presence of an inhibitor whose activity is reversed by addition of IGF-I. Thus, it seems that regulation of growth by vitamin C involves IGF-I and an inhibitor of IGF-I action.

In an effort to develop methods for the early detection of granulocytic leukemia, Dr. Warren Evans is studying the complex process leading to granulocyte maturation. During the past year he and his colleagues have demonstrated the presence of a granulocyte maturation factor (GMF) in human serum which appears to be different from granulocyte stimulating factor and transferrin. Neither of these hematopoietic factors can substitute for GMF. Human transferrin, which potentiates the effect of GMF, appears to play a secondary role in granulocyte maturation. The purification and characteriztion of human GMF are underway.

PROTEINS AND THE CONTROL OF CELLULAR PROCESSES

Proteins and their role in the regulation of cellular processes are studied by three different groups.

Dr. Klee and her colleagues have focused their attention on the role of calcium in the regulation of stimulus response coupling, with particular emphasis on the mechanism of action of calmodulin. The unique characteristic of this protein is its ability to interact with many different target proteins. This group has previously shown that calmodulin interaction with and activation of different targets involves different parts of the calmodulin molecule. In collaboration with Dr. Beckingham of Rice University, they are using calmodulin mutants with altered calcium binding properties to further characterize the role of calcium ions in these interactions and thereby demonstrate that calmodulin can act as a modulator of the calcium signal. The identification of the calmodulin-binding domains of the target proteins, a logical counterpart of this work, is also being carried out. Limited proteolysis of the calmodulin-regulated protein phosphatase, calcineurin, has allowed the isolation of its calmodulin-binding domain and revealed the presence of an inhibitory domain, distinct from the calmodulin-binding domain, whose inhibitory effect is prevented by calmodulin binding. Peptide sequence data are being used to isolate cDNA clones of the two subunits of calcineurin and to synthesize peptide inhibitors of calcineurin which will be used to study the physiological role and mechanism of the Ca^{2+} regulation of this enzyme.

Dr. Wagner and his colleagues have concentrated their efforts on the study of the role of myosin phosphorylation in the control of cellular motility in non-muscle cells. Using myosin from thymus they have obtained strong evidence that the stimulation of actomyosin ATPase is not a direct consequence of myosin light chain phosphorylation but is related to the regulation of myosin filament assembly. Thus, if the unphosphorylated myosin in vivo is filamentous, the cell must use a mechanism different from myosin light phosphorylation to regulate its interaction with actin. The identification of this alternative on/off switch will be studied next.

The group led by Dr. Mage is concerned with the molecular mechanism of immune reactions. The poorly understood interaction between MHC (major histocompatibility complex) molecules and the antigen-specific receptors of T cells plays an important role in many immunological reactions such as tumor immunity and development of vaccines. Dr. Mage and his colleagues are developing methods for the detection of binding of MHC to T cell receptors. Synthetic conjugates of anti-MHC antibodies and water soluble polymers ("polybodies") are used to present MHC antigens to T cells in a multivalent, unhindered configuration. This strategy is meant to increase affinity and permit the detection of the complex. Binding specificity has been demonstrated by inhibition of the reaction with the appropriate MHC antigens. As an outgrowth of this work, they have synthesized conjugates of protein antigens and soluble polymers ("polygens"). These are potentially useful for the measurement of low affinity interactions with cells, and as vaccines. Polybodies will be used as tools for tumor targetting with MHC antigens.

THE ORGANIZATION OF THE HUMAN GENOME

Mapping of human genes and DNA sequences onto human chromosomes has major clinical and biological implications.

Dr. O. Wesley McBride and his colleagues have devoted their efforts to this problem. A large panel of well characterized human-rodent somatic cell hybrids has been established and Southern analysis of the hybrid cell DNAs with cloned probes allows localization of these sequences to specific human chromosomes. Many investigators at NIH and elsewhere who have isolated DNA clones are collaborating with Dr. McBride to map protooncogenes and genes involved in neurological and autoimmune diseases, transcriptional and cellular regulation, carcinogen and drug metabolism, and neoplastic chromosomal breakpoints. In addition to chromosomal localization, information is obtained about the organization and size of the genes and

the presence of multigene families or pseudogenes. In situ hybridization of metaphase chromosome spreads with the cloned probes permits regional localization of these sequences on a chromosome. The probes are also routinely used to detect the presence of restriction fragment length polymorphism (RFLPs) at these sites, providing a complementary method for mapping by genetic linkage analysis. A third method involves mapping by genetic linkage studies of kindreds with nucleic acid probes which detect RFLPs. An important application of theses techniques is the mapping of disease genes for which the underlying defect is unknown and probes are not available. This method has become a major research tool in this laboratory, and it has been used to map disease loci such as those of the syndrome of thyroid hormone resistance and multiple endocrine neoplasia type I.

The efforts of Dr. Maxine Singer's group to characterize the highly repeated, interspersed LINE-1 family in the human genome has, for some years, proceeded from a speculative model: LINE-1s are a family of human transposable elements. This past year, observations in other laboratories demonstrated that LINE-1 elements are transposable and mutagenic in humans, and that transposition causes disease (Hemophilia-A). This finding, as well as the recent recognition that the LINE-1 family is representative of an unusual class of transposable elements that occurs in many eukaryotes, emphasizes the importance of understanding the transposition mechanism. Dr. Singer and her colleagues have taken a broad approach to this problem. They have shown that LINE-1 RNA is a functional mRNA in vitro; they have demonstrated that the 5' leader (900 bp) of full length LINE-1s has promoter activity; antiserum raised against a predicted LINE-1 peptide was shown to react specifically with a 108 KDa peptide in human teratocarcinoma cells suggesting the presence of LINE-1 encoded polypeptide in human cells. Thus evidence for expression of one or more LINE-1s is accumulating.

DNA REPLICATION

The three groups involved in the study of the mechanism and control of DNA replication have been particularly productive this year.

Studies of P1 plasmid maintenance, undertaken by Drs. Michael Yarmolinsky and Dhruba Chattoraj in the Developmental Biochemistry and Genetics Section, substantiate a postulated complex role for the product(s) of the plasmid repA gene in both initiating and limiting DNA replication from the plasmid origin. An understanding of the role of host components in P1 plasmid maintenance is expected to be furthered by the study of recently isolated host mutants that selectively affect the stability of this plasmid. A biochemical analysis of components of the P1 partitioning apparatus has led to the identification of integration host factor (IHF) as a component of that apparatus. IHF serves to increase the affinity of one of two plasmid-encoded partitioning proteins for the P1 centromere. P1 plasmid was shown to be addictive in the sense that loss of the plasmid leads to cellular filamentation and death. Thus, the rare instances of plasmid loss, that are caused by occasional under-replication or by partition errors, do not result in viable cured cells.

Dr. Lichten, a new member of the laboratory, has initiated a research project aimed at understanding the molecular mechanism of meiotic recombination in the yeast, S. cerevisiae. Two major approaches are being followed: An intermediate step in recombination is thought to involve the formation of heteroduplex DNA molecules, where the two strands are of different parental origin. Dr. Lichten developed a technique allowing detection of heteroduplex DNA molecules which contain base-pair mismatches. Using this technique he was able to determine the specificity of base-pair mismatch correction and to determine which strands are transferred during meiotic recombination to form heteroduplex DNA, and the direction in which these strands are transferred. The role of meiotic transcription and regional changes in chromatin structure in the control of meiotic recombination is another approach being taken by Michael Lichten.

Mammalian DNA replication is the focus of the work done by the group led by Samuel Wilson. Earlier success in cloning a cDNA of human β-DNA polymerase has led to the isolation of human genomic DNA spanning the 5'-end of the beta-polymerase gene. Characterization of this DNA defined the transcription start site and the nucleotide sequence of the 100 bp core promoter. The isolated core promoter functions well in transient expression assays in HeLa cells and is strongly activated by products of the adenovirus Ela-Elb oncogene. Kinetic studies of HIV reverse transcriptase identified early steps in the DNA synthesis reaction mechanism. The enzyme initially binds to the oligonucleotide primer, rather than to the template or template-primer complex. The high affinity (200-fold stronger than binding to the normal primer) of the enzyme for a primer analog with a sulfur substituted for a non-bridge oxygen at each nucleotide may help to develop specific inhibitors of this enzyme. The study of the A1 hnRNP protein is facilitated by expression of individual domains of the protein in E. coli. This approach revealed that the glycine/phenylalanine-rich COOH-terminal domain, when studied as a purified peptide, binds tightly to nucleic acids.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00333-24 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
S. M. Wilson	Biologist	LB	NCI
M. G. Mage	Immunochemist	LB	NCI
O. W. McBride	Chief, Cellular Regulation Section	LB	NCI

COOPERATING UNITS *(if any)*

Hematology, Oncology Section, Walter Reed Army Medical Center

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

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 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.

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

Project Description

Objectives:

Our research is aimed at identifying the humoral factors and clarifying the molecular processes that control the appearance of specific subcellular components associated with the differentiation of bone marrow granulocyte precursors. Such information should be useful in developing therapeutic approaches for reversing the arrested differentiation of granulocyte precursors in chromic and acute granulocytic leukemia.

Major Findings:

A. Factors in Human Serum Affecting Human Neutrophil Precursor Maturation

Studies in collaboration with Dr. Jana Bednarek, Dr. Robert Knight of the Walter Reed Army Hospital are aimed at defining the nature of the factors in human serum that induce bone marrow granulocyte end-stage maturation. Previously, we found that human serum contains an inhibitor that blocks the induction of granulocyte alkaline phosphatase, an enzyme which appears after the completion of nuclear folding and cytoplasmic granule formation, morphological maturation changes characteristic of the end stage of granulocyte formation. We find that dialyzed human serum induces end-stage morphological maturation of bone marrow granulocyte precursors and this induction is proportional to the serum dose in the range of 1.0% to 5.0% in the assay medium. At serum concentrations greater than 5.0% a very pronounced inhibition of end-stage maturation is observed. Passage of serum through a DEAE-fractogel column equilibrated with 0.01 M phosphate buffer (pH 7.0) results in the binding of the end-stage granulocyte maturation factor (GMF) to the column. This activity can be eluted from the column with 0.5 M NaCl in a fraction that contains about 7% of the serum protein and is free of inhibitor as judged by the dose response curve for this fraction which reaches a saturating level above 5.0% serum equivalent and shows no inhibitory response above this dose. Using this dose response curve we have assigned the dose of this fraction capable of inducing a half maximal response as being equal to 50 units of GMF activity. Based on this definition of GMF unit of activity, whole serum contains a minimum of 50,000 units of GMF per ml and has a specific activity of approximately 1000 units per mg of serum protein. The partially purified GMF fraction obtained from the DEAE-fractogel column has a specific activity of 7000 units/mg protein representing a 7-fold purification relative to whole serum. Our data indicate that GMF is a protein since it is inactivated by treatment with protease at 37° for 4 hrs.

We showed previously that the maturation-inducing activity of guinea pig serum for guinea pig bone marrow granulocyte precursors could be attributed to the transferrin fraction of this serum and that human serum transferrin could also substitute for guinea pig transferrin as a maturation inducer for guinea pig cells. Our studies with human bone marrow granulocyte precursors indicate, however, that GMF activity in human serum does not appear to be associated with transferrin since neither human nor guinea pig transferrin

alone is capable of inducing human granulocyte maturation. Furthermore, we find that GMF in human serum fails to bind to an anti-human transferrin monoclonal antibody immunoabsorbent column under conditions where transferrin is removed from the serum. Our data does show, however, that purified transferrin greatly potentiates the effect of GMF suggesting that transferrin plays a supporting role in the end-stage maturation of human granulocytes.

GMF also appears to be distinct from human granulocyte colony-stimulating factor (h-G-CSF) which has been shown by others to stimulate granulocyte proliferation at the committed stem cell level of differentiation. Using a wide range of concentrations of recombinant h-G-CSF, we could not detect any maturation-inducing activity with this factor under assay conditions that gave optimal end-stage granulocyte maturation by GMF. Our size exclusion HPLC (SE-HPLC) studies indicate that GMF is in the excluded fraction obtained from a SE-HPLC column having an upper exclusion limit of approximately 300,000 Daltons. Thus, GMF apparently is a much larger molecule than h-G-CSF which has been reported to have a molecular weight of 23,000 Daltons. Attempts are now underway to purify GMF to homogeneity so that a specific immunochemical assay can be developed for this new maturation factor.

B. Defective Phenotypic Protein Pattern of Mature Granulocytes Associated with a Myeloproliferative Syndrome in a Patient with Monosomy 7 in Bone Marrow Cell Precursors

Previously we showed that mature granulocytes obtained from the blood of a patient with a chronic myelocytic leukemia-like syndrome associated with a chromosomal defect ($7q^-$) in the bone marrow are essentially devoid of their lactoferrin content relative to normal granulocytes. Studies in collaboration with Dr. Harry Malech and Dr. Karen Lamox of NIH, using a DNA probe for the lactoferrin gene and mRNA, indicate that this defect is most likely due to a defect in the translation rather than in the transcription of the lactoferrin mRNA since transcripts of the latter were abundantly present in the patient's granulocytes. These data also indicate that the apparent absence of lactoferrin in these cells, which apparently are derived from monosomy 7 precursors, cannot be attributed to the deletion of the lactoferrin gene.

C. Role of Oncogenes in the Pathogenesis of Chronic Myelogenous Leukemia (CML)

Previous studies showed that the N-ras oncogene is associated with the transformation of NIH-3T3 cells into tumorigenic cells by genomic DNA from leukocytes obtained from our guinea pig model for human CML. Work continues in collaboration with Dr. O.W. McBride on the isolation and cloning of this oncogene from normal and leukemic cells (see Annual Report of Dr. O.W. McBride for details).

Publications:

Bednarek JM, Knight RD, Taylor G, Evans WH. Progressive loss of phenotypic proteins in mature granulocytes before the onset of blast crisis in human chronic myelogenous leukemia, JNCI 1988;80:251-7.

Appendix: NIH Contact No. 1-60-25423

Funding: General NCI Contract for FCRC - No individual project funding breakdown available.

Man Years Purchased: 1

Services Performed:

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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00366-17 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 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
M. Falzon	Visiting Associate	LB	NCI

COOPERATING UNITS *(if any)*

E. Leiter, Jackson Laboratory, Bar Harbor, ME

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	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.)*

Mouse intracisternal A particles (IAPs) are defective retroviruses encoded by members of a large family of homologous but non-identical endogenous proviral elements. IAPs are seen in preimplantation mouse embryos, some normal tissues (notably the thymus) and in many mouse tumor cells. Transpositions of IAP elements have caused insertional mutations at both the germ line and somatic cell levels. It is not known whether, or how, particular IAP elements are selectively activated in a given cell type. We are examining the regulation of IAP expression in two ways: (1) through biochemical analysis of site-specific nuclear protein interactions with a cloned IAP LTR known to be promoter competent in vivo; and (2) molecular genetic analysis of IAP activation in thymus cells of inbred mouse strains that differ greatly in their levels of IAP gene expression. The first approach has defined five protein-binding sites in the LTR region upstream of the CAAT and TATA boxes, and revealed that nuclear extracts from different cell lines vary widely in the absolute and relative amounts of binding proteins. Factor concentrations were higher in cells transformed by nuclear oncogenes than those that were not, and correlated well with the relative promoter activity of the LTR when transfected into the corresponding intact cells. IAP expression is known to be sensitive to CpG methylation in the LTR. We have now shown that methylation affects protein binding to one of the five identified sites. In the thymus study, we are determining the number of different IAP transcripts in cells of high and low producer strains by partial sequencing of cDNA clones prepared with IAP-specific primers, and developing methods for the cloning of the corresponding transcriptionally active genomic elements. We are also examining the mechanism by which IAP expression is suppressed in F1 progeny of crosses between high and low IAP producer mouse strains. Collaborative studies are also continuing on the possible role of IAP protein expression in the pathogenesis of a genetically determined mouse diabetes.

GPO 914-918

Project Description

Objectives:

To study the regulation of endogenous retroviral gene expression in relationship to normal development and cellular function, neoplastic transformation and other disease states.

Major Findings:

A. Dr. M. Falzon has continued to study the sequence-specific binding of nuclear proteins to a cloned IAP LTR. We have previously shown that this LTR (subcloned from a 7kb genomic provirus designated MIA14) was active in promoting transcription of a linked CAT gene when transfected into a variety of cell types, and that promoter activity was sharply reduced when three HhaI restriction sites in the upstream regulatory region of the LTR were methylated in vitro before transfection (see 1987 Annual Report of this project). Dr. Falzon has used DNaseI and ExoIII footprinting to identify protein-binding domains in the most 5' 150bp of the LTR. Five binding sites were found, currently designated as a through e in the 5' to 3' direction. Three of the sites contain sequence motifs resembling those of known transcription factor binding sites, namely SP-1 (site a), core enhancer (site c) and AP-1 (site e). A fourth site (d) is separated from the core enhancer by an 11bp alternating purine-pyrimidine stretch which has a theoretical potential for Z-DNA configuration under appropriate salt conditions. This "Z-DNA" region contains one of the three HhaI sites mentioned above. When the 150bp LTR fragment was methylated with HhaI-specific bacterial methylase, the binding of nuclear factors was significantly reduced at several points in site d (assayed by ExoIII digestion) but not detectably changed at the other binding sites. To assess the functional significance of the methylation effect on protein binding at site d, Dr. Falzon prepared a truncated form of the IAP LTR which lacked binding sites a and b and contained only the single HhaI site located between sites c and d. The truncated LTR promoted CAT activity in transfected cells at 40% of the level given by the intact LTR. Methylation of the HhaI site reduced the remaining activity by an additional factor of 75%. This result suggests that the known inhibitory effects of LTR methylation on IAP expression may be mediated at least in part by changes in protein-binding at site d.

   Dr. Falzon used synthetic oligonucleotides based on the LTR sequence to assess the levels of proteins capable of binding to the individual sites. Binding was detected by a mobility shift assay. She found major differences in the absolute and relative amounts of binding proteins in nuclear extracts from various cells. Extracts of cells transformed by nuclear oncogenes (SV40, adenovirus E1a+E1b, c-myc) were comparatively rich in factors that reacted with the IAP LTR, regardless of the cell type or species of origin. In general, the levels of binding proteins in the nuclear extracts correlated well with the relative promoter activity of the IAP LTR when transfected into the corresponding intact cells.

B. Collaborative studies with Dr. E. Leiter have suggested that autoimmunity to IAP-related antigens may have a role in pathogenesis of genetically deter-

mined mouse diabetes (see previous Annual Reports of this project). Mice of certain inbred strains express IAPs in the insulin-producing beta cells of their pancreatic islets, and also have circulating antibodies against IAP proteins. IAP expression is augmented in beta cells of these strains when islets are explanted and cultured in medium containing high glucose levels comparable to those found in the serum of mice with the db/db genotype. Under in vivo conditions, an increased expression of IAP-related antigens may help to target an autoimmune attack on the beta cells, leading to cell death and permanent diabetes. We plan to test this hypothesis by creating transgenic mice carrying a functional IAP coding region under transcriptional control of an insulin promoter rather than its own LTR. Other constructs with this promoter have given constitutive expression of the linked genes in beta cells of the transgenic mice. We have inserted the coding region of a cloned and sequenced 7kb IAP element (MIA14) into a vector containing the insulin promoter. A large quantity of purified plasmid has been provided to Dr. Leiter for the biological studies. He is currently testing the plasmid for ability to encode IAP proteins by transfecting it into rat insulinoma cells, where the insulin gene promoter is known to be active. If this control is satisfactory, he will attempt to obtain transgenic mice of a strain which ordinarily does not express IAPs in its beta cells. The aim is to determine first whether IAP proteins are constitutively expressed in the beta cells of the transgenic animals and second whether such animals exhibit an IAP-mediated autoimmune reaction when they are made homozygous for the db gene.

C. J. Mietz has begun a study of the strain-specific regulation of IAP expression in mouse thymus. We reported previously that IAP-specific RNAs and IAP protein synthesis rates varied greatly in the thymuses of young mice from different inbred strains. In addition, thymus IAP expression was suppressed in F1 mice derived from crosses between BALB/cJ (high IAP producers) and C57BL/6J (low producers). The binding studies described in section A have shown that IAP transcription is regulated by multiple cellular factors interacting with binding sites on the IAP LTRs. Variations in IAP expression probably reflect differences in the amounts and proportions of factors that can also affect a number of other genes. We wish to ascertain (1) whether the same or different IAP elements (which can be recognized as cDNA clones by virtue of their individual patterns of random base changes) are expressed in the thymuses of different mouse strains, (2) what factors determine the selective activation of certain elements within the large IAP gene pool. Finally, we hope to elucidate the mechanism of F1 suppression mentioned above, since this may reflect a more general mechanism for gene regulation.

Publications:

Mietz JA, Grossman Z, Lueders KK, Kuff EL. Nucleotide sequence of a complete intracisternal A-particle genome: relationship to known aspects of particle assembly and function, J Virol 1987;61:3020-9.

Kuff EL, Lueders KK. The intracisternal A-particle gene family: structure and functional aspects, Adv Cancer Res 1988;51:183-276.

Kuff EL, Lueders KK. Structure and evolution of retroviruses with homology to the transposable intracisternal A-particle elements of mice. In: Kingsman AJ, Chater KF, Kingsman SM, eds. Transposition. Cambridge: University Press, 1988;247-70.

Serreze DV, Leiter EH, Kuff EL, Jardieu P, Ishizaka K. Molecular mimicry between insulin and retroviral antigen p73: development of cross-reactive antibodies in sera of NOD and C57BL/KsJ-db/db mice, Diabetes 1988;37:351-8.

Jensen L, Kuff EL, Wilson SH, Steinberg AD, Klineman DM. Antibodies from patients and mice with autoimmune diseases react with recombinant hnRNP core protein A1, J Autoimmun 1988; in press.

Lueders KK, Kuff EL. Transposition of intracisternal A-particle genes. Prog Nucleic Acid Res Mol Biol 1988; in press.

Kuff EL. Factors affecting transposition of intracisternal A-particle proviral elements. Proceedings of the Banbury Conference, vol 30. New York: Banbury Center, 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00945-15 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
J. Palka	Visiting Fellow	LB	NCI
E. Schalk	Guest Researcher	LB	NCI
V. Shah	Visiting Fellow	LB	NCI
K. Hadley	IRTA Fellow	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Biological Interactions Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	4	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 had shown that ascorbic acid deficiency in guinea pigs resulted in decreased levels of IGF-I. They also suggested that there was formation of a circulating inhibitor of DNA synthesis in 3T3 cells and of extracellular matrix synthesis in cultured chondrocytes. Experiments in which serum from scorbutic or fasted guinea pigs was mixed with serum from normal guinea pigs confirmed that an inhibitor is present in the experimental sera. In both cell systems, inhibition could be reversed by the addition of IGF-I to the cultures containing scorbutic or fasted sera. The results support our model in which effects of vitamin C deficiency on growth occur because of the fasting state caused by the deficiency. Regulation of growth under these conditions probably results from decreased levels of circulating IGF-I as well as the appearance of an inhibitor of IGF-I activity.

We have continued studies to elucidate the mechanisms responsible for the unusual collagen phenotype of 4-nitroquinoline-1-oxide transformed Syrian hamster embryo fibroblasts. These cells fail to synthesize the proα 1(I) subunit of type I procollagen but continue to synthesize altered forms of the other subunit, proα 2 (I). Present experiments using cell-free translation and hybridization of RNA from normal and transformed fibroblasts with labeled proα 1 (I) DNA probes showed that mRNA of proα 1 (I) is absent from the transformant. Southern blotting of cellular DNA indicated that the gross structure of the proα 1 (I) gene was unchanged by transformation. The polypeptide translated by RNA from the transformant was similar to proα 2 (I). These studies provide a molecular basis for the transformed collagen phenotype.

Project Description

Objectives:

The objectives of this project are to elucidate the mechanisms of collagen secretion and to determine what humoral factors regulate the rates of synthesis of the extracellular matrix components collagen and proteoglycan in animals.

Major Findings:

A. The Role of Ascorbate in Connective Tissue Metabolism

We have proposed a model in which the effects of ascorbate deficiency on the synthesis of collagen and proteoglycan in connective tissues of guinea pigs are equivalent to the effects of fasting. In the model, these effects are independent of the role of ascorbate in proline hydroxylation. Our previous results showed that sera from scorbutic and fasted guinea pigs contained decreased levels of IGF-I (somatomedin C) and the results also suggested that these sera contained a circulating inhibitor of DNA synthesis in 3T3 cells. Direct evidence for such an inhibitor has been obtained by carrying out experiments in which scorbutic and fasted sera were added to cultures containing normal guinea pig serum. In the case of 3T3 cells, the experimental sera inhibited DNA synthesis, as measured by the incorporation of [^{3}H] thymidine. We also tested the effects of these sera on chick embryo chondrocyte cultures and found that scorbutic serum inhibited both collagen and proteoglycan synthesis. Quiescent 3T3 cells require only EGF, PDGF and IGF-I for stimulation of DNA synthesis, and since we add saturating amounts of the first two factors in the assay system, it seemed likely that the inhibitor was antagonistic to IGF-I. Therefore, we added IGF-I to 3T3 cultures containing scorbutic or fasted sera and found that inhibition of DNA synthesis was reversed. Similarly, addition of IGF-I to chondrocyte cultures containing scorbutic serum reversed the inhibition of collagen and proteoglycan synthesis. The results suggest that the inhibitor might be an IGF-I binding protein and we are currently investigating this possibility.

Previous pulse-chase experiments indicated that the reduction in collagen production in chondrocytes cultured in scorbutic serum was caused by decreased synthesis rather than increased degradation. In our present studies, we have determined whether decreased collagen synthesis results from decreased levels of mRNA for cartilage collagen (type II). We isolated RNA from chondrocytes cultured in either normal or scorbutic guinea pig serum and carried out a dot-blot hybridization assay using a radioactive type II collagen cDNA probe. There was approximately 30% as much mRNA in the cells cultured in scorbutic serum compared to cells cultured in normal serum. The extent of the decrease in mRNA closely parallels the effect of scorbutic guinea pig serum on collagen synthesis in chondrocytes. In cells treated with scorbutic serum but also receiving IGF-I, the level of mRNA remained normal. Thus, it appears that IGF-I is required to maintain optimal levels of collagen mRNA and that the putative inhibitor prevents the interaction between IGF-I and chondrocytes.

B. Regulation of Collagen Synthesis in Chemically Transformed Syrian Hamster (NQT-SHE) Fibroblasts

Our previous results showed that Syrian hamster embryo fibroblasts transformed by 4-nitroquinoline-1-oxide failed to synthesize the proα 1(I) subunit of type I procollagen but continued to synthesize altered forms of the other subunit, proα 2(I). This was unusual, since synthesis of the two subunits generally is coordinately regulated. Present experiments using cell-free translation and hybridization of RNA from normal and transformed fibroblasts with labeled proα 1(I) DNA probes show that mRNA for proα 1(I) is absent from the transformant. In contrast, dot-blot and Southern blot hybridizations of cellular DNAs with proα 1(I) DNA probes demonstrated that the transformed cells contained sequences for the gene and that the gross structure of the gene was unchanged by transformation. mRNA for the other type I procollagen subunit, proα 2(I), was present in transformed cells and the major collagenous polypeptide translated from this RNA migrated like the normal proα 2(I) chain during sodium dodecyl sulfate polyacrylamide gel electrophoresis. The translated procollagen chain was cleaved to an α2(I) sized collagen chain by pepsin at 4°C. These studies provide a molecular basis for the observed collagen phenotype of NQT-SHE cells.

Publications:

Tschank G, Hanauske-Abel HM, Peterkofsky B. The effectiveness of inhibitors of soluble prolyl hydroxylase against the enzyme in the cisternae of isolated microsomes, Arch Biochem Biophys 1988;261:312-23.

Majmudar G, Schalk E, Bateman J, Peterkofsky B. Uncoupled expression of mRNAs for α1(I) and α2(I) procollagen chains in chemically transformed Syrian hamster fibroblasts, J Biol Chem 1988;263:5555-9.

Chojkier M, Flaherty M, Peterkofsky B, Majmudar GH, Spanheimer RG, Brenner DA. Different mechanisms decrease hepatic collagen and albumin production in fasted rats, Hepatology 1988; in press.

Oyamada I, Bird TA, Peterkofsky B. Decreased extracellular matrix production in scurvy involves a humoral factor other than ascorbate, Biochem Biophys Res Commun 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05202-21 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
A. Bale	Biotechnology Fellow	LB	NCI
S. Olson	Biotechnology Fellow	LB	NCI
P. Burnett	Biologist	LB	NCI
M. Wang	Visiting Associate	LB	NCI
A. Mitchell	Chemist	LB	NCI

COOPERATING UNITS *(if any)* J. Minna et al., D. Hatfield, F. Gonzalez, M. Bustin, Hon-Sum Ko, L.M. Staudt, NCI; Sue Goo Rhee, NHLBI; S. Usala, B.D. Weintraub et al., S.J. Marx et al., J. Chan, L. Kohn et al., NIDDKD; R. Eldridge et al., NINCDS; A. Notkins et al., NIDR; E.E. Strehler, Zurich; J. Idle, London

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.4	4.0	1.4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Analysis of interspecific somatic cell hybrids segregating human chromosomes permits the localization of human genes to specific chromosomes. We have previously constructed a large panel of human-rodent hybrids and used them to chromosomally map protooncogenes and various other human genes by Southern analysis of hybrid cell DNAs with cloned DNA probes. In collaborative studies, these hybrids have now been used to chromosomally map several additional proto-oncogenes and putative chromosomal neoplastic breakpoints, multiple tRNA and cytochrome P450 genes, and a variety of genes for regulatory, structural, and enzyme proteins. Current techniques allow construction of genomic restriction maps of genes and pseudogenes and usually permit assessment of copy number. The probes are simultaneously used to identify restriction fragment length polymorphism (RFLPs) in DNAs from normal individuals; these RFLPs are used in subsequent mapping studies by genetic linkage analysis and this is a major portion of the current research effort. In collaborative studies with the B. D. Weintraub lab, close linkage has been found between the syndrome of generalized thyroid hormone resistance (GTHR) and the human c-erbAβ gene indicating that a defect in this thyroid hormone receptor probably underlies the disease in some cases. Other collaborative studies have established linkage of the MEN1 (multiple endocrine neoplasia, type 1) disease gene to the INT 2 locus on chromosome $11q_{13}$.

In studies designed to detect the involvement of β-polymerase or Poly (ADP-ribose) polymerase in diseases associated with DNA repair defects, no abnormalities of these genes have been found in xeroderma pigmentosum and ataxia telangiectasia by Southern and Northern analyses. The use of a more discriminating RNase protection assay have been unsuccessful and a genetic approach will probably be required.

Project Description

Objectives:

1) Human chromosomal mapping of protooncogenes and genes involved in DNA synthesis, carcinogen metabolism, and regulation of cell proliferation and gene expression, and understanding role of these genes in human neoplasia, 2) mapping the genes for hereditary cancer predisposition syndromes, and 3) determining the role of DNA polymerase and poly(ADP-ribose) polymerase in DNA repair defects and neoplasia.

Major Findings:

A. 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. It also allows regional mapping on chromosomes (using translocations or spontaneous breaks), detection of pseudogenes and multigene families, determination of gene copy number, identification of restriction fragment length polymorphism (RFLPs) detected by probes at these sites, and detection of cloned chromosomal breakpoints in neoplasms. A large series of human rodent somatic cell hybrids have previously been isolated, characterized, and hybrid cell DNAs used to chromosomally map a large group of protooncogenes, immunoglobulin genes and pseudogenes, tRNA genes and pseudogenes, neoplastic break points, and genes for structural, regulatory, and enzyme proteins using cloned DNA probes in collaboration with several groups. Procedures have been developed to distinguish functional genes from processed pseudogenes, detect multiple homologous sequences on a single chromosome, and identify RFLPs at these chromosomal loci. Southern blots have been prepared recently from a large panel of somatic cell hybrids DNAs separately digested with 11 different restriction endonucleases thereby permitting the chromosomal identification of specific polymorphic (RFLP) loci using probes which detect sequences on multiple chromosomes. Subfragments of cloned cDNAs have been used increasingly as probes to allow construction of genomic restriction maps of uncloned functional genes and asyntenic pseudogenes. This has significantly facilitated cloning of functional gene fragments and pseudogenes from chromosome-specific libraries and provides a rapid method for the identification of both gene and pseudogene sequences cloned from genomic libraries. RFLPs detected in our studies are useful in current genetic linkage studies involving several human diseases including cystic fibrosis, multiple endocrine neoplasia, type II (MEN2), Neurofibromatosis II (NF2), Alzheimer's disease, and Wilson's disease.

We have recently mapped several additional cytochrome oxidase genes including P450PCN1, P450IVA2, P450IIB1 and IIB2, and P450 cytochrome reductase in collaboration with F. Gonzalez and NMOR-1 and NMOR-2 with A. Jaiswal. Some other genes chromosomally localized have been glutathione peroxidase and two GPX processed pseudogenes (D. Hatfield), phospholipase C2 (S.G. Rhee), a Ca^{++} dependent ATPase (E. E. Strehler), HMG17 (M. Bustin), several L-*myc* pseudogenes (J. Minna), a lymphoid-restricted regulatory protein gene oct-2

(L. M. Staudt), calcineurin B (C. Klee),four tRNA gene clusters (R. Pirtle) and a putative translocation breakpoint in a lymphoma (H. S. Ko). The genes for three cDNAs encoding autoantigens in Grave's disease have been mapped including one which appears to be a functional TSH receptor (and its 5 processed pseudogenes) in collaboration with J. Chan, L. Kohn, M. Lerman, and A. Notkins.

B. Genetic Linkage Mapping in Kindreds with Nucleic Acid Probes which Detect Restriction Fragment Length Polymorphisms (RFLPs)

This method provides information complementary to that obtained by the physical mapping studies. It also allows chromosomal mapping, and ultimately cloning and identification, of genes for human genetic diseases in which the underlying defect is currently unknown. We are collaborators in the Centre d'Etude du Polymorphism Human (C.E.P.H.) human gene mapping project and we have received DNA samples from the CEPH pedigrees for linkage analysis. Utilization of this panel requires digestion of DNAs from about 400 individuals with about 12 different restriction endonucleases and preparation of high quality Southern blots on membranes after fractionation of DNA fragments by agarose gel electrophoresis. This process is well underway while attempting to maximize the output of useful information from blots which have been prepared. Two point and multipoint linkage analysis programs are being used with a microcomputer to analyze data. Allen Bale has also been involved in several gene linkage projects in collaboration with other laboratories.

1. Genetic linkage between the syndrome of generalized thyroid hormone resistance (GTHR) and the human c-erbAß gene (collaborative study with S.J. Usala, B.D. Weintraub, C. Weinberger et al.). Two asyntenic c-erbA genes encode proteins which appear to be thyroid hormone receptors and the hypothesis that a defect in the c-erbAß receptor might be involved in this autosomal dominant disorder was tested by genetic linkage analysis (LIPED program) of one extensively studied kindred. DNAs from normal individuals digested with 12 different restriction endonucleases were first screened for RFLPs with the c-erbAß probe. Using RFLPs detected with Bam H1 and Eco RV, DNAs from all 17 members of the GTHR kindred were examined by Southern analysis with the c-erbAß probe and all individuals were fully informative. Linkage analysis demonstrated a maximum lod score between the GTHR and c-erbAß loci of 3.91 at a recombination fraction of 0. These data indicate that the gene for GTHR is closely linked to, or identical with, the c-erbAß gene on human chromosome 3.

2. Physical and genetic linkage map of 6 markers on chromosome 13. Six "anonymous" DNA probes isolated and characterized in this laboratory identify polymorphic loci on human chromosome 13. A physical linkage map of these markers has been constructed using somatic cell hybrids retaining only portions of this chromosome and a genetic linkage map of these markers has been obtained by analysis of families in collaboration with Drs. A. Bowcock and L. Cavalli-Sforza (Stanford U.). Several of these markers have been genetically linked to the disease gene in Wilson' disease.

3. Multipoint linkage analysis of a large MEN1 (multiple endocrine neoplasia, type 1) kindred (in collaboration with S. Bale, S. Marx, J. Mulvihill, and J. Auerbach). Four probes (INT2, PGA, D11S33, D11S84) which detect RFLPs on chromosome 11 have been used for Southern analysis of a large MEN1 kindred. Multipoint linkage analysis permitted localization of the disease gene in close proximity (i.e. 3 centimorgans) to the INT2 locus on the proximal long arm (11q13) of chromosome 11. Dr. Allen Bale is currently attempting to further localize the disease gene by analysis of DNAs from tumors of these patients for loss of heterozygosity of each of the four linked DNA markers.

4. Nevoid Basal Cell Carcinoma. Dr. Allen Bale has clinically studied several kindred with this syndrome and established EBV-transformed lymphoblastoid cell lines and isolated DNAs. Initial studies have failed to establish genetic linkage of the disease gene to any RFLP markers tested. There is some cytogenetic evidence that the locus for this disease may be on chromosome 15. Therefore, a large number of chromosome 15 RFLP markers will be used to genetically map the disease locus on chromosome 15 or to exclude its presence on this chromosome.

5. Neurofibromatosis II. Dr. Roswell Eldridge has clinically studied several kindred with NF2. Drs. G. Rouleau, J. Gusella et al. have performed genetic linkage studies on one NF 2 kindred and shown linkage of the disease locus to an "anonymous" DNA probe on chromosome 22q12 but the available probes were noninformative in another NF2 kindred. We have identified several RFLPs on chromosome 22q which are detected by cDNA probes which we have chromosomally mapped with other collaborators; these include a TSH receptor and a P450dbl cDNA probes. We are undertaking studies in collaboration with Drs. G. Rowleau, B. Watson, R. Eldridge et al. that are designed to genetically map these new loci on chromosome 22q and to use these new markers for genetic linkage to the disease locus in the second NF2 kindred.

C. Chromosomal Mapping By in situ Hybridization of Metaphase Spreads.

This method is being used in combination with the other mapping methods in an effort to obtain precise physical localization of genes on chromosomes. We are currently mapping some of the tRNA gene loci by all three methods (i.e. somatic cell hybrid analysis, in situ hybridization, and genetic linkage studies).

D. Analysis for Involvement of DNA Polymerase Beta or Poly(ADP-ribose) Polymerase in DNA Repair Defects.

1. About 50 EBV transformed lymphoblastoid lines derived from patients with xeroderma pigmentosum (6 different complementation groups) or ataxia telangiectasia and relatives have been obtained from the NIGMS Human Genetic Mutant Cell Repository; DNA has been isolated from mass cultures, digested with 12 different restriction endonucleases, electrophoretically size fractionated, and transferred (Southern) to nylon mem-

branes. No molecular rearrangements were detected by Southern hybridization with a 560 bp human ß-polymerase 5' cDNA probe and a 699 bp ß-pol 3' probe. Initial results suggested the possibility of linkage disequilibrium between an infrequent RFLP allele in the 5' flank of the ß-Pol gene and one complementation group of XP but analysis of additional DNAs from XP fibroblast lines did not support this interpretation. These same blots were probed with a full length poly (ADP-ribose) polymerase (Pol-P) cDNA probe. The same RFLPs were detected in these DNAs as have been found in DNAs isolated from normal individuals. However, a potential Pol-P rearrangement was found in both Bgl II and Eco RV digests of DNAs from one XP group C patient and his mother and brother. Therefore, Bgl II and Eco RV digests of DNAs isolated from a larger group (39 total) of "normal" individuals were screened with this probe and the same Bgl II and Eco RV pattern was found in one individual. Thus, it currently appears that this represents an infrequent allele (i.e. variant) rather than a Pol-P rearrangement in the XP family.

2. More sensitive methods have been used to screen for Pol-P and ß-Pol defects in these patients. Northern analysis of RNAs isolated from the XP patients with ß-Pol probes disclosed the expected 1.1 kb mRNA. Quantitative changes in the mRNA levels cannot be excluded at present.

3. An antisense ß-Pol riboprobe was prepared and used in a RNase protection assay after hybridization with RNAs isolated from the XP patient's cells. Although protection of the probe was obtained in control experiments after hybridization with a sense-strand unlabeled ß-Pol riboprobe, no-protection was obtained with RNA isolated from XP cells or normal cells. Various control experiments have failed to provide an explanation for the failure of the RNase protection assay and further studies are in progress.

4. A 12.6 kb Eco RI genomic fragment containing the ß Pol exons 1 and 2 has been mapped with restriction enzymes and it is apparent that a 4-5 kb insertion sometimes occurs 1-1.5 kb upstream from the transcription start site resulting in RFLPs detected with several enzymes. However, recent studies by S. Widen and S. Wilson indicate that all regulatory sequences for ß-Pol are found within about 100 bp of the transcription start site. Hence, it seems unlikely that the insertion is functionally significant.

5. It currently appears that a genetic approach for detection of defects in in ß-Pol and Pol-P in these diseases involving DNA repair defects will be required. Additional RFLPs in flanking DNA segments will be sought for these studies.

Publications:

McBride OW, Umeno M, Gelboin HV, Gonzalez FJ. A Taq I polymorphism in the human P450 IIE1 gene on chromosome 10 (CYP2E), Nucleic Acids Res 1987;15:10071.

Thayer RE, Harper ME, Sawyer J, Singer MF, McBride OW. Localization of unique DNA sequence to band p16 of human chromosome 4, Cytogenet Cell Genet 1987;45:75-9.

Kimura S, Kotani T, McBride OW, Umeki K, Hirai K, Nakayama T, Ohtaki S. Human thyroid peroxidase: complete cDNA and protein sequence, chromosome mapping, and identification of two alternately spliced mRNAs, Proc Natl Acad Sci USA 1987;84:5555-9.

McBride OW, Rajagopalan M, Hatfield D. Opal suppressor phosphoserine tRNA gene and pseudogene are located on human chromosomes 19 and 22, respectively, J Biol Chem 1987;262:11163-6.

Okino ST, Quattrochi LC, Pendurthi UR, McBride OW, Tukey RH. Characterization of multiple human cytochrome P-450 1 cDNAs, J Biol Chem 1987;262:16072-9.

Gonzalez FJ, Schmid BJ, Umeno M, McBride OW, Hardwick JP, Meyer UA, Gelboin HV, Idle JR. Human P450PCN1: Sequence, chromosome localization, and direct evidence through cDNA expression that P450PCN1 is nifedipine oxidase, DNA 1988;7:79-86.

Skoda RC, Demierre A, McBride OW, Gonzalez FJ, Meyer UA. Human microsomal xenobiotic epoxide hydrolase: complemetary DNA sequence, complementary DNA-directed expression in Cos-1 cells, and chromosomal localization, J Biol Chem 1988;263:1549-54.

Gonzalez FJ, Vilbois F, Hardwick JP, McBride OW, Nebert DW, Gelboin HV, Meyer UA. Human debrisoquine 4-hydroxylase (P450IID1): cDNA and deduced amino acid sequence and mapping of the P450 IID1 gene to chromosome 22, Genomics; in press.

Umeno M, McBride OW, Yang CS, Gelboin HV, Gonzalez FJ. Human ethanol-inducible P450 II E 1: Complete gene sequence, promoter characterization, chromosome mapping, regulation, and cDNA directed expression, J Biol Chem; in press.

Jaiswal AK, McBride OW, Adesnik M, Nebert DW. Human dioxin-inducible NAD(P)H: menadione oxidoreductase: cDNA sequence and localization of gene to chromosome 16, J Biol Chem 1988;263; in press.

Brooks BA, McBride OW, Dolphin CT, Farrall M, Scambler PJ, Gonzalez FJ Idle JR. Cytochrome P450 PCNI (nifedipine oxidase) is tightly linked to collagen type I alpha on 7q21.3-q22, Am J Hum Genet; in press.

Bale AE, Weinberger C, McBride OW. A retinoic acid receptor cDNA probe identifies a moderately frequent RFLP on chromosome 17, Nucleic Acids Res; in press.

Bale AE, Usala S, Weinberger C, McBride OW. A cDNA probe from the hc-erbAß gene on chromosome 3 detects a high frequency RFLP, Nucleic Acids Res; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05203-20 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
B. Nardelli	Visiting Fellow	LB NCI
U. Grohmann	Guest Worker	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 20892

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.)*

Our goal is the development of methods for 1) the specific isolation of antigen-reactive cells for the study of the molecular interactions that occur in their immune reactions, and 2) their application to problems of tumor immunology. In order to be able to directly measure binding of antigen-specific receptors to MHC molecules, we have accomplished the synthesis of conjugates of anti-MHC antibodies and water-soluble polymers. In the course of our studies of molecular interactions of antigen-reactive cells as they relate to problems of tumor immunology, we have accomplished improved syntheses of soluble antigen-polymer conjugates ("polygens") and of soluble antibody-polymer conjugates ("polybodies"), and have partially characterized these conjugates with respect to size, composition, and activity, and have also improved the technique for their synthesis. In studies aimed at understanding the enhanced immunogenicity of a mutagenized ("xenogenized") cell line, we have, with the aid of polyclonal and monoclonal antibodies, partially characterized a cell surface antigen of the xenogenized cell line not found on the original (parental) tumor line.

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 the development of new techniques and reagents for their immuno-chemical 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 (CTL_p) 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 phenomena such as graft rejection and the graft-versus-host reaction.

Major Findings:

A. Improved Synthesis and Characterization of Conjugates of Antibodies and Water-soluble Polymers ("Polybodies") For Use in Presenting MHC Antigens to T cells, and in Measuring the Affinity of the Interaction

Understanding the nature of the binding of MHC molecules to the antigen-specific receptors of T cells is central to many immunological problems, both in tumor immunity and vaccine development. In order to present MHC antigens to these cells in multivalent unhindered configuration and to directly measure binding and activation, we have improved our synthesis of "polybodies" and have made estimates of their size (about 700 kD for monomer) and in the millions for larger units, and have found, on a mass basis, that they retain immunological activity comparable to the unconjugated monomeric IgG antibodies. Their specificity has been demonstrated by inhibition of reaction with cells by the appropriate MHC antigens.

B. Synthesis of "Polygens" for Possible Use as Tools for Measurement of Low Affinity Interactions, and as Vaccines

"Ovalbumin polygen" - a conjugate of monomeric ovalbumin and an activated linear soluble polyacrylamide, and been synthesized, partially characterized, and, in preliminary results, has been found to be immunogenic in mice in the absence of adjuvant.

C. Antibodies Specific for Xenogenized Cells React with a Cell Surface Antigen of Xenogenized Cells

"Xenogenization" (i.e. mutagenization of tumor cells to increase their immunogenicity) is a potential approach to cancer immunotherapy of tumors that fail to elicit an effective immune response. In collaboration with the Institute of Pharmacology of the University of Perugia (supported by Italy-USA joint project CNR (Italy) contract number N.86.0031.04),

additional polyclonal and monoclonal antibodies have been prepared and used to partially identify a cell surface antigen of the xenogenized cells (in collaboration with Drs. Ettore Appella and Steve Ullrich of the Laboratory of Cell biology, DCBD, NCI).

D. Tumor Targeting by Attachment of Antigens

We have further characterized heteroconjugates of different monoclonal antibodies to MHC antigens and found (as was the case with polybodies) that the reaction with cell-bound MHC molecules is specifically inhibitable by soluble MHC molecules.

Publications:

Romani L, Grohmann U, Puccetti P, Nardelli B, Mage MG, Fioretti MC. Cell-mediated immunity to chemically xenogenized tumors, II. Evidence for accessory function and self-antigen presentation by a highly immunogenic tumor variant, Cell Immunol 1988;111:365-78.

Romani L, Grohmann U, Fazioli F, Puccetti P, Mage MG, Fioretti MC. Cell-mediated immunity to chemically xenogenized tumors, I. Inhibition by specific antisera and H-2 associativity of the novel antigens, Cancer Immunol Immunother; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05214-17 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

DNA Synthesis in Mammalian Cells: Structure and Function of DNA Polymerases

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

S. H. Wilson	Medical Officer	LB	NCI
B. Zmudzka	Visiting Associate	LB	NCI
P. Kedar	Visiting Fellow	LB	NCI
S. Widen	IRTA Fellow	LB	NCI
J. Abbotts	IRTA Fellow	LB	NCI

COOPERATING UNITS *(if any)* A. Fornace, K. Williams, Yale; R. Karpel, University of Maryland; J. Collins, Virginia Commonwealth University; A. Matsukage, Aichi Can. Cen., Nagoya, Japan; J. Siedlecki, Inst. Oncology, Warsaw, Poland

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.9	3.5	.4

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 continued biochemical studies of mammalian DNA replication proteins. A cDNA for β-polymerase has been subcloned in expression vectors; the protein has been overproduced in E. coli and purified in mg quantities. In vitro DNA repair activities of the recombinant enzyme are being studied. Genomic DNA spanning the gene for human β-polymerase was further characterized. The promoter region was studied by detailed deletion mutagenesis using a transient expression system. In other work, we found that β-polymerase mRNA in cultured rodent cells is induced by DNA alkylating agents, but not by other DNA damaging agents. A tissue survey indicated a higher level of the mRNA in testes than other tissues of the rat.

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

Project Description

Objectives:

The ultimate objective of this research program is to understand mechanisms of DNA synthesis in mammalian cells. Our main approaches are enzymology of DNA synthesis proteins and examination of DNA replication *in vitro* using purified polymerases and other required proteins.

It is anticipated that these studies on the properties, specificities, and regulation of DNA replication proteins will, in conjunction with results from other laboratories, help answer important questions about mechanisms and control of DNA replication in the mammalian cell.

Major Findings:

A. Structure and Function of Mammalian DNA Polymerases

Our current approach toward the general problem of understanding the function and biochemistry of mammalian DNA polymerases is 1) characterization of polymerase cDNAs and genes, 2) analysis of expression of polymerase mRNAs and 3) studies of enzyme proteins overproduced in *E. coli* using cloned cDNAs.

1. Human β-polymerase

DNA polymerase β (β-pol) is a housekeeping enzyme considered to be involved in DNA repair in vertebrate cells. We cloned a fragment of genomic DNA spanning the first two exons of the human β-pol gene and ~11 kb of the flanking region. The segment just 5' of the transcription start site can direct expression of the bacterial chloramphenicol acetyltransferase (CAT) gene in HeLa cells. A sequence containing only 113 bp of flanking DNA has promoter activity, and various constructs containing up to 4.8 kb of flanking sequence are expressed at a similar level, indicating that with this assay the important regulatory elements are located within or proximal to the ~100 bp core promoter. S1 nuclease mapping was used to show that transcription of the transfected genes is initiated at the same position as the endogenous β-pol gene. The region upstream of the transcription start site is G+C rich and contains neither CAAT nor TATA boxes, but does have three decanucleotide elements matching high affinity binding sites for the RNA polymerase II transcription factor Sp1. Extending 5' from position -40 and surrounded by Sp1 consensus binding elements, there is a 10 nt sequence with perfect dyad symmetry, GTGACGTCAC. Similar sequences are found in a number of cellular and viral promoters, including several adenovirus promoters. Experiments to test whether the core β-pol promoter is activated by the adenovirus early region products showed that cotransfection with an adenovirus expression plasmid strongly activates expression of the β-pol promoter.

The open reading frames predicting the 335 amino acid proteins of human and rat were subcloned into the *E. coli* expression plasmid pRC23. After induction of transformed cells the crude soluble extract contained an

abundant new protein corresponding to β-polymerase. The rat protein has been purified to homogeneity in 1 to 2 g quantities. Catalytic properties of the recombinant β-polymerases are being studied using defined template•primer systems. The enzymes also are being studied by physical techniques.

2. β-polymerase mRNA in Chinese hamster cells

In prokaryotes it is well documented that induction of DNA repair genes is central in the response to DNA damage. In mammalian cells, however, only a few of the genes involved in DNA repair have been isolated, and induction of a DNA repair gene in response to DNA damage has not yet been established. DNA polymerase β (β-polymerase) has a synthetic role in the repair of DNA damage in vertebrate cells. We showed that β-polymerase mRNA is induced in hamster cells after treatment with DNA alkylating agents. No induction was seen after treatment with other agents. These results suggest that the β-polymerase gene may be part of a DNA damage-inducible DNA repair system in mammalian cells.

3. β-polymerase in RNA in rat tissue

A cDNA probe for rat β-polymerase was used to measure the level and size complexity of β-pol mRNA in regenerating rat liver and other rat tissues. A slight increase in β-polymerase mRNA was observed 18-24 hours after partial hepatectomy. In most adult rat tissues (liver, heart, kidney, stomach, spleen, thymus, lung and brain) the abundance of β-polymerase mRNAL is low. In contrast, young brain and testes exhibited mRNA levels 10 and 20 times higher than other tissues. There was a correlation between the observed levels of β-polymerase mRNA and β-polymerase enzymatic activity reported in literature. Two dominant (4.0 and 1.4 kb) and two minor (2.5 and 2.2 kb) transcripts hybridizing to the β-polymerase probe were found in all tissues examined.

4. Mouse chromosomal localization of β-polymerase gene

In collaboration with O.W. McBride, genomic DNA from mouse-hamster hybrids was subjected to Southern blot analysis using β-polymerase cDNAs as probes. The results to date indicate that the β-polymerase gene is single copy and is located on mouse chromosome 8. This is consistent with our earlier results on the chromosomal location of the β-polymerase gene in humans.

Publications:

Anderson RS, Lawrence CB, Wilson SH, Beattie KL. Genetic relatedness of DNA polymerase beta and terminal deoxynucleotidyltransferase, Gene 1987;60:163-73.

Abbotts J, SenGupta DN, Zmudzka B, Widen S, Notario V, Wilson SH. Expression of human DNA polymerase beta in E. coli and characterization of the recombinant enzyme, Biochemistry 1988;27:901-9.

Nowak R, Kaczmarek BZ, Wilson SH, Siedlecki JA. Tissue specific expression of DNA polymerase β mRNA in rat. Biochem Biophys Acta; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05231-14 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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, Protein Biochemistry Section	LB	NCI
R. Ochia	Guest Researcher	LB	NCI
M. H. Krinks	Chemist	LB	NCI
D. Guerini	Visiting Fellow	LB	NCI
Wei Xi Qin	Visiting Fellow	LB	NCI
M. P. Strub	Visiting Fellow	LB	NCI
J. Mackall	Guest Researcher	LB	NCI

COOPERATING UNITS *(if any)* Dr. J. Shiloach, NIAMMD; Dr. P. Cohen, Univ. of Dundee, Scotland; Dr. H. Plattner (Univ. of Konstanz, FRG); Drs. D.J. Tash and A.R. Means (Baylor Univ., Houston, TX); Dr. K. Beckingham, (Rice Univ., Houston, TX); Dr. W. Hahn, (Colorado Health Science Center, Denver, CO); Dr. H. Gainer, NINCDS

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Protein Biochemistry

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.5	6.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The mechanism of stimulus response coupling mediated by Ca^{2+} and calmodulin was studied at two levels: 1) We demonstrated that the cooperative binding of calcium to calmodulin, leading to a stepwise conformational change, is due to an allosteric transition affecting the amino and carboxyl-terminal halves of the molecule. Binding of two mol of Ca^{2+} decreases the affinity for the two remaining sites. The central helix which connects the two halves of the molecule plays a critical role in this negative cooperativity. Mutant calmodulins with altered Ca^{2+}-binding properties are being studied to elucidate the role of this structural change in calmodulin function. 2) Studies of the interaction of calmodulin with calcineurin, a calmodulin-stimulated protein phosphatase, have included the isolation and sequencing of the cDNA clones of the two subunits of the enzyme. The complete human cDNA clone of the small subunit was isolated and sequenced. The mapping and amino acid sequence of three of the functional domains of the large subunit (catalytic site, calmodulin-binding and inhibitory domains) was affected by protein sequencing. The sequence data are being used to aid the isolation and complete sequencing of the human calcineurin A gene.

GPO 914-918

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 the mechanism of the regulation of the Ca^{2+}-dependent stimulation of the protein phosphatase, calcineurin, by calmodulin. These studies are undertaken to elucidate the roles of the two second messengers, Ca^{2+} and cAMP, in the regulation of cell function.

Major Findings:

A. Calmodulin and Calcium Regulation of Cellular Activity

1. Regulation of intracellular calcium levels

This project was terminated after the departure of Thierry Jean from the Laboratory.

2. Interaction of calmodulin with calcium

These studies were undertaken to obtain insight into the molecular mechanism of the Ca^{2+}-dependent activation of many different enzymes by calmodulin. Binding of Ca^{2+} to calmodulin is a highly cooperative process leading to the stepwise conformational changes essential for the interaction of calmodulin with the proteins under its control. Ca^{2+} binding to the two adjacent Ca^{2+}-binding domains which constitute both the carboxyl and amino-halves of calmodulin exhibits positive cooperativity (Hill coefficients 1.8-2). Binding of Ca^{2+} to the sites on one half of the molecule decreases the affinity of the other half for Ca^{2+}. The central helix connecting the two halves of the molecule is required for this negative control which is amplified by physiological concentrations of Mg^{2+} (Dianne Newton and Julia Mackall). In collaboration with Kathleen Beckingham we have now shown that a single mutation in a Ca^{2+} binding domain not only decreases the affinity of this domain for Ca^{2+} but also affects the affinity of the remaining three sites, thereby demonstrating the tight functional link between the four Ca^{2+}-binding domains of calmodulin. These mutants, with altered Ca^{2+}-binding properties, will be used to dissect the role of calmodulin in the Ca^{2+} stimulation of enzyme activity.

3. Interaction of calmodulin with target proteins

Our recent studies have been focused on the regulation of the calmodulin-stimulated protein phosphatase, calcineurin. The regulation of this enzyme by Ca^{2+} is mediated by two different Ca^{2+}-binding proteins: an integral subunit of the enzyme, calcineurin B, and calmodulin. Previous studies by Michael Hubbard had shown that the catalytic unit of the enzyme, calcineurin A, is composed of four distinct functional domains: an inhibitory domain, calmodulin-binding

and calcineurin B-binding domains and a catalytic site. The mapping of these functional domains along the primary structure of calcineurin A has now been accomplished by protein sequencing methods combined with limited proteolysis. Synthetic peptides corresponding to the inhibitory and calmodulin-binding domains will be used to further elucidate the functional properties of these domains and to prepare specific inhibitors of the enzyme. Oligonucleotides corresponding to the amino and carboxyl-termini of calcineurin A were synthesized in order to identify human cDNA clones of calcineurin A. The complete sequence of the protein is being determined by sequencing of the cDNA clones (Danilo Guerini, Wei Xi Qin and Marie Krinks in collaboration with Philip Cohen). The human cDNA coding for the Ca^{2+}-binding subunit, calcineurin B, was isolated using a 234 bp probe encompassing part of the coding sequence of the mouse protein isolated by antibody screening of a lambda gt11 cDNA library. The 2700 bp clone contains the complete coding region, a 700 bp untranslated sequence 5' to the initiator ATG codon and a 1300 bp 3' untranslated sequence ending with a putative polyadenylation signal. The length of the cDNA is similar to that of rat calcineurin B mRNA determined by Northern blot analysis. The sequence of the human protein is identical to that of the bovine protein, with three exceptions: substitution of Met11 and Ser153 by cysteines and an additional valyl residue at the COOH-terminus. This work was done in collaboration with John Sikela and William Hahn (University of Colorado Health Sciences Center) who isolated the mouse cDNA probe.

The two subunits of calcineurin and appropriately selected mutants will be expressed in E. coli to study the reconstitution of the enzyme and elucidate the roles of calcineurin B and calmodulin in the Ca^{2+} regulation of calcineurin. Selection of mutation sites will be based on the limited proteolysis data as well as crosslinking studies of Marie Paule Strub designed to identify the interaction sites on the two subunits of calcineurin and calmodulin. Chromosome mapping of the calcineurin genes is presently being done in collaboration with Dr. McBride of this laboratory.

4. Physiological roles of calcineurin

Two model systems are currently being used to ascertain the physiological role of calcineurin. The involvement of calcineurin in the control of flagellar motility in dog sperm is being studied in collaboration with Dr. J. Tash (Baylor University) and its role in exocytosis is being investigated in Paramecium in collaboration with Dr. Plattner (University of Konstanz, FRG).

B. cAMP-Dependent Protein Kinase in Drosophila Melanogaster

This project was terminated upon the departure of Dr. John Foster.

Publications:

Hubbard MJ, Klee CB. Calmodulin binding by calcineurin. J Biol Chem 1987;262:15062-70.

Momayezi M, Lumpert CJ, Kersken H, Gras U, Plattner H, Krinks MH, Klee CB. Exocytosis induction in Paramecium tetraaurelia cells by exogenous phosphoprotein phosphatase in vivo and in vitro. Possible involvement of calcineurin in exocytotic membrane fusion, J Cell Biol 1987;105:181-9.

Draetta G, Klee CB. Purification of calmodulin-dependent phosphodiesterase with calmodulin fragment 1-77 linked to sepharose, Methods Enzymol 1988;159:573-81.

Klee CB, Krinks, MH, Hubbard MJ. The domain structure of calcineurin. In: Norman A, Vanaman T, Means AR, eds. Calcium binding proteins in health and disease. San Diego: Academic Press, 1988;481-90.

Klee CB, Draetta G, Hubbard MJ. Calcineurin, Adv Enzymol 1988;61:201-302.

Means AR, Klee CB. Summary of workshop on Ca^{2+}-binding proteins, Adv Cyclic Nucleotide Protein Phosphorylation Res 1988;21; in press.

Tash JS, Krinks MH, Means RL, Klee CB, Means AR. Identification, characterization and functional correlation of calmodulin-dependent protein phosphatase in sperm: association with dynein. J Cell Biol 1988; in press.

Krinks MH, Klee CB, Pant HC, Gainer H. Identification and quantification of calcium-binding proteins in squid axoplasm. J Neurosci 1988; in press.

Newton DL, Krinks MH, Kaufman JB, Shiloach J, Klee CB. Large scale preparation of calmodulin, Prep Biochem 1988; in press.

Klee CB. Ca^{2+}-dependent phospholipid-(and membrane)-binding proteins, Biochemistry 1988; in press.

Klee CB. Calmodulin: Ca^{2+} binding and interactions with target proteins. In: Cohen P, Klee CB, eds. Molecular aspects of cellular regulation. Amsterdam: Elsevier, 1988; in press.

Klee CB, Cohen P. Calmodulin Regulated Protein Phosphatase. In: Cohen P, and Klee CB, eds. Molecular Aspects of Cellular Regulation, vol 5. Amsterdam: Elsevier, 1988; in press.

Jean T. The release of calcium from endoplasmic reticulum: effect of inositol 1,4,5-trisphosphate and guanine nucleotides. Proceedings of the 12th European symposium on hormone and cell regulation. Mont St. Odile, France: 1987; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05244-11 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Organization of Repeated DNA Sequences in Primates

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

M.F. Singer	Scientist Emeritus	LB	NCI
R.E. Thayer	Chemist	LB	NCI
T. Fanning	Expert	LB	NCI
V. Krek	Guest Researcher	LB	NCI
G. Swergold	PRAT Fellow	LB	NCI
J. Carr	Guest Researcher	LB	NCI

COOPERATING UNITS *(if any)*

Lisa Forman, National Zoo; Hector Seuanez, NCI-Frederick; Bill Modi, NCI-Frederick

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.8	5.5	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.)*

The LINE-1 family of interspersed repeated sequences is ubiquitous in mammals. Genetic analysis (by others) has demonstrated that in humans, LINE-1 elements are transposable and mutagenic. Lacking the terminal repeats characteristic of most known classes of prokaryotic and eukaryotic transposable elements, mobility of the LINE-1 family (and related elements in invertebrates) must involve unique mechanisms. This work aims to understand LINE-1 transposition in human cells. Experimental approaches are informed by the following widely-discussed model. One or more of the approximately 4000 full length LINE-1 elements in the human genome are transcribed and translated. Among the translation products is a reverse transcriptase that reverse transcribes LINE-1 RNAs. The resulting cDNAs are inserted into randomly located staggered cleavages in genomic DNA. In support of this model, we have demonstrated that 1) the 900 bp long untranslatable 5' end of some 6 kbp human LINE-1 sequences has promoter activity in human and monkey cells permitting synthesis of a marker gene product (chloramphenicol transacetylase) fused downstream; 2) the 5' ends of a small subset of LINE-1 elements are hypomethylated in NTera2D1 cells (a teratocarcinoma line) the only human cell type in which polyadenylated, cytoplasmic LINE-1 DNA has been observed; 3) anti-serum raised against a synthetic peptide predicted by LINE-1 ORF2 (the ORF that predicts a polypeptide with reverse transcriptase homology) recognizes a trpE/ORF 2 fusion protein made in E. coli and a 108 kDa polypeptide associated with the 100,000 x g pellet of the cytoplasmic fraction of Ntera2D2 cell extracts.

Repeated DNA sequence probes are being used to study genome evolution and species identity in New World primates.

Project Description

Objectives:

Earlier speculation suggested that the LINE-1 family of interspersed repeats, found in all mammals tested, represents a transposable element. Data on humans (acquired by others in the past year) demonstrates the validity of this speculation. Human LINE-1s are transposable in somatic cells and perhaps during meiosis or early development. They can be mutagenic, causing human disease, as shown by 2 independent occurrences of (factor VIII) Hemophilia-A. Our main objective is to understand the mechanism and control of transposition. Our working model is that at least one of the approximately $5X10^4$ family members in the human genome is transcribed and translated to yield a reverse transcriptase which converts LINE-1 RNAs into DNA that is inserted in essentially random genomic sites. This year we initiated a variety of approaches to this objective. We also use LINE-1 and other repeated sequences as tools for the study of evolutionary biology.

Major Findings:

A. The LINE-1 Family

Last year we reported on the overall features of cloned cDNAs representing, full length polyA$^+$, cytoplasmic LINE-1 transcripts in human teratocarcinoma cells (cell line Ntera2D1). Sequencing indicated that the transcribed LINE-1s belong to a distinctive subset, recognizable by a consensus DNA sequence at the 3' end. We speculated that this transcribed subset might represent 'active' elements, a speculation supported when the LINE-1 sequences associated with human factor VIII gene mutagenesis, were shown to belong to the distinctive subset. In contrast, among 7 cDNA clones analyzed, none showed a 5' region that differed significantly from the genomic consensus.

The absence of terminal repeats on LINE-1 elements places them in an unusual class of transposable elements. An important question is: what is the location of transcriptional regulatory elements that can promote RNA synthesis? One hypothesis is that the regulatory elements are within the unit length genomic LINE-1 sequence. The potential of the 5' 900bp of LINE-1 to promote RNA transcription was investigated by fusing it to the chloramphenicol acetyl transferase (CAT) gene and measuring CAT activity in transfected tissue culture cells. Early results indicate significant promoter activity in several cell lines. Promoter activity is greatly diminished by deleting various portions of the segment. We aim to clone the 5' flanking regions of transcribable elements. Two approaches are being taken. First, using as a probe the characteristic DNA sequence at the 3' end of the transcribed units, clones contain full length LINE-1s were isolated from a genomic library. The flanking sequences are now being characterized. The second approach depends on preliminary experiments suggesting that the 5' end of some LINE-1 elements are hypomethylated in NTera2D1 cells compared to other cell lines.

As reported last year, in vitro synthesized RNA corresponding to one of the Ntera2D1 cDNAs appears to be translated, in vitro, to give the polypeptide predicted by LINE-1 ORF1 (the 5' ORF). Evidence that LINE-1 ORF2 is translatable is very scanty. Yet, ORF2 is of great interest because its sequence 1) predicts a polypeptide with homology to reverse transcriptase and 2) is the most highly conserved portion of LINE-1 among mammals and related invertebrate elements. Antibodies were prepared to synthetic peptides predicted by ORF2 and to ß-galactosidase-ORF 2 fusion proteins produced in E. coli from appropriate recombinant DNAs. Each fusion contained a different part of ORF2. The anti-sera were tested for reactivity against extracts of E. coli synthesizing fusion proteins of E. coli trpE gene product and ORF2. One of the antipeptide antibodies reacted specifically with the trpE - ORF2 fusion protein (on Western blots); binding was inhibited by the peptide. This anti-peptide antiserum identified an 108 kDa polypeptide in the 100,000 x g pellet fraction of Ntera2D1 cell extracts. Two of the anti-sera raised against ß-galactosidase-ORF 2 fusion proteins also appear to recognize trpE-ORF2 fusion protein products on Western blots. Correlation of the trpE-ORF 2 fusion proteins recognized by the anti-peptide antisera with those recognized by the two anti-ß-galactosidase/ORF2 antisera is not straight forward. Problems arise because 1) the several antisera are directed against different ORF2 epitopes and 2) multiple trpE/ORF2 fusion products are observed, as expected, from instability (or incomplete translation) in E. coli. However, some bands among the trpE/ORF2 fusion products do appear to interact with all three antisera. TrpE/ORF 2 fusion proteins are being tested for reverse transcriptase activity.

B. Repetitive DNA Sequences as a Tool in Evolutionary Biology

Repetitive DNAs are being used to study carnivore and primate evolution. The sequences of a large number of domestic dog and grey fox satellites suggest that in both species, satellites were homogenized about 2 million years ago and their current modes of evolution differ. The differences, involve the rates at which G•C base pairs are lost from the satellites. Repetitive DNAs from a variety of South American primates have been cloned and characterized in order to study LINE-1 and genome evolution in a primate clade that has evolved independently of the human/great ape lineage for tens of millions of years. Some of the clones distinguish species, subspecies, and individuals within a population and, thus, may be applicable to decisions regarding species conservation projects.

Publications:

Fanning T. Origin and evolution of a major feline satellite DNA, J Mol Biol 1987;197:627-34.

Fanning T, Singer M. LINE-1: A mammalian transposable element, Biochem Biophys Acta 1987;910:203-12.

Maresca A, Thayer RE, Gosselin P, Singer MF. Genomic organization of low copy number sequences that are associated with deca-satellite DNA in the monkey genome, Nucleic Acids Res 1987;15:8799-8813.

Thayer RE, Harper ME, Sawyer J, Singer MF, McBride OW. Localization of a unique DNA sequence to band p16 of human chromosome 4, Cytogenet Cell Genet 1987;45:75-9.

Skowronski J, Fanning T, Singer M. Unit-length LINE-1 transcripts in human teratocarcinoma cells, Mol Cell Biol 1988;8:1385-97.

Singer M, Skowronski J, Fanning TG, Mongkolsuk S. The functional potential of the human LINE-1 family of interspersed repeats in eukaryotic transposable elements as mutagenic agents. Cold Spring Harbor Laboratory Banbury Center. Cold Spring Harbor, New York, 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05258-09 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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. Eldridge	Biochemist	LB	NCI
J. Rodriguez	NRC Associate	LB	NCI
B. Winter	Visiting Fellow	LB	NCI
W. Quitschke	Visiting Fellow	LB	NCI
L. DePonti-Zilli	Visiting Fellow	LB	NCI
Z.-Y. Lin	Visiting Fellow	LB	NCI
C. Dechesne	Visiting Fellow	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Biochemistry of Gene Expression Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8	7	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.)*

Myogenic tissue culture systems provide extremely useful model systems in which to study gene regulation during tissue formation. We have isolated and characterized a variety of muscle-specific and house-keeping genes that are differentially expressed during myogenesis. These include three different actin genes (alpha cardiac, alpha skeletal and beta cytoplasmic actins) and the myosin light chain 1-3 gene. All but the beta cytoplasmic actin gene are expressed only in muscle tissue. The cis acting sequence elements involved in the tissue specific regulated expression of these genes have been characterized and we are currently in the process of identifying proteins that interact with these regulatory regions to modulate gene expression. Mutational analysis of factor binding sites coupled with in vivo and in vitro transcriptional studies allow one to determine the nature of the various regions in these cis regulatory sequence elements. PC12 cells undergo neuronal differentiation in response to nerve growth factor (NGF). We have isolated the complete cDNA and corresponding gene for an mRNA that is specifically induced 50-80 fold by NGF in PC12 cells. Antibodies to the polypeptide, prepared with LacZ fusions to different ORFs in the cDNA, define a protein of 72,000 daltons which appears to be a marker for cells in the CNS that respond to NGF. Preliminary studies with the gene promoter suggest NGF induces transcription. The cis regulatory elements of the gene responsive to NGF induction are under study. Using defined oligo nucleotide probes to highly conserved regions in the myosin heavy chain polypeptide from all the nonmuscle myosins sequenced to date we have isolated the yeast myosin gene. The role of the yeast myosin in the life cycle of yeast is under study. Gene replacement studies with mutant and chimeric myosin genes from Acanthamoeba or the higher vertebrates will give information on the structure-function relationships of the various myosins and a means to map functionally important domains in myosin and actin.

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

Project Description

Objectives:

We would like to understand the mechanisms that control the expression of eukaryotic genes during development and tissue formation.

Major Findings:

A. Regulation of Actin Isoform Expression Alpha Cardiac

Alpha cardiac actin is the major actin isoform expressed in embryonic and cultured muscle. The adult isoform, alpha skeletal actin, eventually replaces the cardiac actin as the muscle matures. During myogenesis expression of the cytoplasmic isoform, beta actin, is repressed even though it is expressed in practically all dividing cells. Using the appropriate deletions we have introduced modified versions of the various actin genes into mouse myogenic cells and have defined the major cis regulatory sequence elements that are required for tissue restricted expression or repression. The alpha actin isoforms require approximately 200 base pairs 5' to the transcriptional start site to maintain the appropriate pattern of regulated expression. Using band shift assays and methylation interference foot-print techniques we have determined the following: (1) the same shift pattern is seen with nuclear extracts from muscle and nonmuscle cells, and (2) the interference foot-prints from all extracts tested on a given actin promoter are identical. At present no "muscle specific" promoter binding proteins have been detected by these procedures. However, each actin promoter exhibits different binding domains for proteins in the nuclear extracts and these binding sites do not compete for the same protein as judged by competition experiments. The G and A nucleotides involved in the binding sites are being mutated in order to determine their role in regulation. The modification of generic factors may be important to the tissue specific expression of the alpha actin genes. The beta cytoplasmic actin gene is regulated by a 50 base pair sequence element next to the canonical polyA addition sequence in the 3' UTR of the gene. This sequence is conserved in all the vertebrate beta actins sequenced to date. Nuclear runon studies indicate this element somehow controls the steady state levels of beta actin mRNA at the transcriptional level. This 3' sequence does not behave like a classical enhancer since it is both orientation and position dependent for its action. This element does not need to be associated with the canonical polyA site in order to regulate beta actin levels and studies are under way to determine the importance of position within the transcribed region of the gene. The element is able to impart the beta actin regulatory pattern to any gene in a muscle cell background. Even though bandshift assays suggest proteins are bound to this sequence element, we have been unable to foot-print successfully any proteins to date. The preparation of muscle nuclei extracts has been difficult due to the large quantities of muscle proteins that interfere with the extraction procedure. Linker scanner studies with this element are under investigation to locate precisely the sequences involved in the regulation.

B. The Myosin Light Chain 1-3 Gene from Chicken

The myosin light chains (1 and 3) are encoded by a single gene with two promoters that are differentially utilized during myogenesis, light chain 1 preceding light chain 3. Each light chain transcript contains both unique and common exons: exons 1 and 4 are unique to light chain 1 and exons 2 and 3 are unique to light chain 3. The unique exons are spliced to common exons 5 through 9. Two transcripts are produced for each light chain due to equal utilization of tandem adenylation signals. The light chain promoters from the chicken gene appear to function only in chicken muscle cells even though there is a very high sequence homology in the light chain promoters from chicken, mouse, and rat. In recent collaborations with Dr. Nadia Rosenthal at Harvard/Massachusetts General, expression of the chicken promoters in rodent cells appears dependent upon an enhancer element in the 3' end of the rat gene. If this enhancer is placed 5' to the chicken light chain promoters the species barrier is eliminated. The enhancer has little or no effect in a chicken muscle background. Deletion analysis on the chicken light chain promoters has defined regions of approximately 200 base pairs 5' to the transcriptional starts that are required for tissue restricted regulated expression of light chain mRNAs. We are currently studying the nuclear proteins that bind to these regions to determine the factors common and unique to each promoter. A mutational analysis will determine if the binding domains are important for regulation during myogenesis.

C. Gene Regulation by Nerve Growth Factor in PC12 Cells

PC12 cells differentiate into neuronal cells in response to nerve growth factor (NGF). We have isolated the complete cDNA and the gene encoding an mRNA that is induced 50-80 fold when PC12 cells are treated with NGF. The longest open reading frame in the cDNA encodes a polypeptide of approximately 72,000 daltons. LacZ fusion proteins containing two different portions of this open reading frame were used to generate polyclonal antibodies in rabbits to study induction and processing of the polypeptide in PC12 cells. The protein is rapidly processed into smaller fragments after an initial cleavage. Immunostaining of adult tissue sections and primary embryonic cultures of neuronal cells from the rat reveals that the protein is present in the adrenal medulla, the dorsal root ganglia, the spinal cord, in specific areas of the hippocampus and in other regions of the CNS. The discrete cleavage products seen in PC12 cells by Western blot analysis, taken together with the unusual number of potential dibasic amino acid cleavage sites in the protein, suggest the protein may be a precursor for smaller peptides, possibly hormones or neurotransmitters, expressed in certain cell populations derived from the neural crest and in neurons within the CNS. This novel protein appears to serve as a marker for NGF responsive cells. Preliminary studies on the promoter of the gene suggest NGF activates transcription. The precise sequence elements involved in this NGF response are under investigation.

D. Studies on the Yeast Myosin Gene

Using defined oligo nucleotide probes to highly conserved regions in the myosin genes of C. elegans, slime mold, Acanthamoeba, and various verte-

brates we have screened a yeast genomic library and have isolated the yeast MyoI gene. This has been determined by sequence analysis and comparison to a recently published partial sequence for MyoI. Gene replacement studies will be used to study the functional equivalence of yeast myosin with other characterized myosin genes, especially the Acanthamoeba myosin I and II genes. Studies are being extended with replacements of the single yeast actin gene with the well characterized vertebrate actin genes. Preliminary results indicate the vertebrate beta actin gene will not substitute functionally for the yeast gene even though the proteins are similar in amino acid sequence and size. This is still under investigation.

Publications:

Hammer III JA, Bowers B, Paterson BM, Korn E. Complete nucleotide sequence and deduced polypeptide sequence of a non-muscle myosin heavy chain gene from Acanthamoeba: evidence of a hinge in the rod-like tail, J Cell Biol 1987;105:913-25.

Zehner ZE, Li Y, Roe BA, Paterson BM, Sax CM. The chicken vimentin gene: nucleotide sequence, regulatory elements and comparison to the hamster gene, J Biol Chem 1987;262:8112-20.

Quitschke WW, Eldridge JD, Paterson BM. Tissue specific expression of a sarcomeric and cytoplasmic actin does not depend on a particular CAAT sequence element. In: Siddiqui MAQ, Cullen B, Gage P, Skalka A-M, Weissbach H, eds. Mechanisms of control of gene expression. New York: A R Liss Inc., 1988;59-69.

Arnold HH, Lohse P, Paterson BM, Winter B. The regulated expression of chicken muscle genes transfected into myogenic culture cells. In: Siddiqui MAQ, Cullen B, Gage P, Skalka A-M, Weissbach H, eds. Mechanisms of control of gene expression. New York: A R Liss Inc., 1988;85-96

DePonti-Zilli L, Seiler-Tuyns A, Paterson BM. A 40-base-pair sequence in the 3' end of the beta actin gene regulates beta actin mRNA transcription during myogenesis, Proc Natl Acad Sci USA 1988:85;1389-93.

Billeter R, Quitschke W, Paterson BM. Approximately 1 kilobase of sequence 5' to the two myosin light-chain 1f/3f gene cap sites is sufficient for differentiation-dependent expression, Mol Cell Biol 1988:8;1361-5.

Arnold HH, Tannich E, Paterson BM. The promoter of the chicken cardiac myosin light chain 2 gene shows cell-specific expression in transfected primary cultures of chicken muscle, Nucleic Acids Res 1988;16:2411-29.

Quitschke WW, Eldridge JD, Paterson BM. Analysis of regulatory regions in the alpha cardiac actin promoter. In: Kedes L, Stockdale F, eds. Cellular and Molecular Biology of Muscle Development. New York, A R Liss Inc., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05262-08 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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)*

D. H. Hamer	Research Chemist	LB	NCI
J. Imbert	Visiting Fellow	LB	NCI
M. Ernoult	Visiting Fellow	LB	NCI
P. Furst	Guest Researcher	LB	NCI
R. Hackett	Staff Fellow	LB	NCI
S. Hu	Chemist	LB	NCI
V. Culotta	Guest Researcher	LB	NCI
X. Cao	Visiting Fellow	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Biochemistry of Gene Expression Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.0	7.0	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.)*

The metallothioneins provide a useful model to study how eukaryotic genes are regulated and how the gene products allow cellular adaptation and homeostasis. Using the simple unicellular eukaryote Saccharomyces cerevisiae, a detailed analysis of the cis-acting control sequences that regulate metallothionein gene transcription in response to copper has been carried out. In addition, a trans-acting gene required for transcriptional induction has been identified and cloned. We propose that regulation of the yeast gene occurs through the interaction of both positive and negative factors. Structure-function relationships of yeast metallothionein have been studied by a combination of site-specific mutagenesis and biophysical techniques and residues critical to the functional activity of this protein have been identified. Metallothionein gene regulation in the more complex cells of the mouse has also been analyzed. Systematic point mutagenesis of the control region reveals different residues required for induction and for efficient transcription. Two factors that bind to these regions have been identified and separated.

Projection 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 conditions.

Major Findings:

A. Yeast

1. Control Sequences

Copper ions strongly induce transcription of the yeast copper metallothionein gene, CUP1. A short DNA element that contains the upstream copper control region has been synthesized and individual point mutants in 37 of its 44 basepairs have been analyzed for transcription. Two major classes of mutant phenotype were observed: down mutants that are non-inducible by copper, and up mutants, that have a high basal level of expression. The down mutants, which presumably represent changes in the binding site for an activator protein, are clustered in the 5' portion of the control region. The up mutants, which may define a repressor binding site, are located in the 3' portion of the element.

2. Trans-Activator

In order to isolate mutants in the activator protein, a mutagenized yeast population was screened for cells that were copper sensitive and incapable of inducing CUP1 gene transcription in the presence of copper ions. Dr. Dennis Thiele, who initiated this project in my laboratory and now has his own group at U. of Michigan, cloned the gene corresponding to one of these mutations. Constructed mutants in this gene are noninducible for CUP1 expression but otherwise normal. The behavior of CUP1 up and down mutants in the trans-acting mutant background should clarify our model for regulation by the concerted action of an activator and a repressor.

3. Structure-Function

A series of truncation and point mutants of the yeast metallothionein gene have been constructed. The mutant proteins have been tested for function by transformation of a $CUP1^{-}$ yeast strain and for structure by copper analysis, proteolysis, EXAFS, UV luminescence and antibody reactivity. Suprisingly, different cysteine residues have markedly different effects on formation of the sulfur-copper multinuclear complex despite their chemical equivalence. In order to determine whether these mutations define nucleation sites for cooperative folding of the protein, we are collaborating with other groups to determine the dynamic and static structures of the protein by NMR and x-ray crystallography.

B. Mouse

1. Control Sequences

Transcription of the mouse metallothionein-I gene is strongly induced by heavy metals such as cadmium. We showed previously that induction is mediated by a short DNA sequence, the MRE, repeated 5 times in the upstream flanking region. To pinpoint essential residues within this 19 basepair region, we constructed and tested the transcription competence of 15 individual point mutants either as monomers or dimers. Sequences in the 5' portion of the element, which are strongly conserved amongst higher eukaryotic MREs, are essential for heavy metal induction. Sequences in the 3' portion, which are homologeous to the binding site for the ubiquitous factor Sp1, are not required for induction but do influence the efficiency of transcription. The MREs regulate transcription in a cooperative fashion that depends on their precise spacing.

2. Binding Factors

We previously reported an activity in total nuclear extracts that binds to MREd. Attempts to purify this activity by conventional and DNA affinity chromatography suggest that at least two proteins are involved, one of which resembles Sp1. These proteins, which have now been cleanly separated, are being studied for their affinity to the wild-type and mutated MRE oligonucleotides.

Publications:

Thiele DJ, Wright CF, Walling MJ, Hamer DH. Function and regulation of yeast copperthionein. In: Kagi JHR, ed. Metallothionein II: Experientia Supplementum, vol 52. Basel: Birkhauser Berlag, 1987;423-9.

Wright CF, McKenny K, Hamer DH, Byrd J, Winge DR. Structural and functional studies of the amino terminus of yeast metallothionein, JBC 1987;262:12912-9.

Leone A, Hamer DH. Abnormal copper metabolism and regulation of metallothionein gene expression in Menkes' disease. In: Kagi JHR, ed. Metallothionein II: Experientia Supplementum, vol 52. Basel: Birkhauser Berlag, 1987;477-80.

Wright CF, Hamer DH, McKenny K. Autoregulation of the yeast copper metallothionein gene depends on metal binding, JBC 1988;263:1570-4.

Byrd J, Bergers RM, McMillin DR, Wright CF, Hamer D, Winge DR. Characterization of the copper-thiolate cluster in yeast metallothionein and two truncated mutants, J Biol Chem 1988;263:6688-94.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05263-07 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters 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 Scientist	LB	NCI
B. Walker	Staff Fellow	LB	NCI
S. Wilson	Research Assistant	LB	NCI
V. Zimarino	Visiting Associate	LB	NCI
J. L. Brown	Visiting Associate	LB	NCI
S. Rabindran	IRTA Fellow	LB	NCI
G. Lavorgna	Visiting Fellow	LB	NCI
B. Davis	Laboratory Worker	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Developmental Biochemistry and Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.5	7.0	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.)*

This group has continued its analysis of sequence-specific DNA-binding proteins in Drosophila, with focus on the heat shock genes and the segmentation gene fushi tarazu. For the heat shock genes, the critical regulatory transcription factor, heat shock activator protein (renamed heat shock factor, HSF) has been purified to homogeneity repeatedly; anti-HSF antibodies have been produced, and candidate gene clones of HSF have been identified in a lambda gt11 expression library. In addition, purification and characterization of the other transcription factor found to bind to the heat shock promoter (at the TATA box) is under way.

A screen for DNA-binding proteins that interact with the "zebra" control element of the fushi tarazu gene that is expressed in alternate segmental primordia early in Drosophila embryogenesis around the blastoderm stage has been completed using nuclear extracts of developmentally staged embryos. At least 3 major proteins or protein complexes were observed to bind specifically to a region encompassing 200 bp upstream of the transcriptional start site. One factor, NF-ftz1 has been purified to homogeneity, antibodies have been generated, and the cloning of its gene is in progress.

The biochemical purification and subsequent cloning of the factors that interact with these genes should provide the important reagents necessary for the next stage of analysis of mechanisms of regulation of cellular homeostasis and of segmental determination.

Project Description

Objectives:

A knowledge of gene regulation is fundamental to an understanding of eukaryotic development and differentiation. We wish to determine general principles of gene regulation through analysis and reconstruction of specific protein-DNA interactions in vivo and in vitro around gene control sequences. Our studies are focused on two Drosophila systems: the heat shock genes and the fushi tarazu (ftz) gene involved in the segmental patterning of the early embryo.

Major Techniques Employed and Major Findings:

A. Studies with Purified Heat Shock Activator Protein

The purification by sequence-specific affinity chromatography of heat shock activator protein, renamed heat shock factor (HSF) has been standardized to a routine 2-week long procedure. In spite of the low abundance of HSF in the cell, sufficient quantities (20 µg) have been obtained to continue a program of immunization to generate mouse polyclonal antibodies that have high specificity for HSF, as measured by the formation of a high mobility antibody-HSF-DNA complex on native gels, and by strong reaction with the 110 kd HSF polypeptide on Western blots. The antibodies also show a weak reaction with mammalian and yeast HSF.

Preparations for microsequence analysis of purified HSF are under way. We have successfully employed SDS-PAGE to separate chromatography, electroblotted the 110 kd HSF polypeptide to PVDF membrane and eluted the protein using detergents SDS/NP-40. Since the amount of HSF available is extremely low, we intend to maximize the informational return from microsequence analysis of the protein by sequencing HSF peptides generated by cyanogen bromide cleavage or specific proteolysis.

We have demonstrated that purified HSF is functionally active with respect to the transcriptional activation of heat shock genes by micro-injection of purified HSF in Xenopus laevis oocyte nuclei that had previously received an injection of a cloned hsp70 gene. The ability of less purified HSF fractions to stimulate hsp70 transcription in Xenopus oocytes in currently being investigated. If successful, this assay will be a useful alternative to the in vitro transcription assay for the analysis of other transcription factors.

B. Cloning the Drosophila HSF Gene

We have used anti-HSF antibodies in several large screens of a lambda gt11 cDNA expression library made from Drosophila embryo RNA. From a total of about 1 million plaques, two promising candidates have tested positive in repeated screens during plaque purification. The insert sizes are 800 bp and 1600 bp, too small to encode a complete 110 kd protein. We are currently using the inserts to screen for the full length cDNA, prior to sequencing the gene and testing its authenticity by expressing a functional protein. In parallel, we shall check the gene sequence against that predicted by the results of protein microsequencing.

C. Inducible and Constitutive HSF in Different Species

By means of a quantitative whole cell extraction procedure and the gel mobility shift assay, HSF has been identified in multiple species, including Drosophila, mouse, human, toad, chicken, and yeast. In all species studied so far excluding yeast, the binding of HSF to the heat shock regulatory sequence is inducible when cells are subjected to heat or chemical shock. This inducible binding occurs in the absence of de novo protein synthesis, and it suggests that HSF is activated by a post-translational modification. In contrast, HSF in the yeast S. cerevisiae and S. pombe has constitutive binding activity, suggesting a different mechanism of heat shock activation in yeast that is based on the activation of HSF already bound to the regulatory sequence but is incapable of starting the transcriptional machinery. The two mechanisms are not mutually exclusive, and it will be most interesting to determine the mechanism of other species as they are studied in other laboratories.

Further divergence in HSF has also been observed in different molecular sizes in yeast, Drosophila, and human. Using a photo-affinity labeling technique developed in this laboratory, the molecular sizes as measured by mobility on SDS-PAGE of yeast, Drosophila and human HSF are 140 kd, 110 kd, and 90 kd, respectively. Although the DNA-binding domain of HSF has presumably been highly conserved in evolution (all HSF's bind to the same DNA sequence) other parts of the protein have probably diverged substantially.

D. DNA Binding Proteins for the Segmentation Gene Fushi Tarazu

A screen for DNA-binding proteins that interact with the "zebra" control element of the fushi tarazu gene that is expressed in alternate segmental primordia around the blastoderm stage has been completed. Nuclear extracts were prepared from Drosophila embryos at defined stages of development, and DNA-binding factors were analyzed by the exonuclease III protection and gel mobility shift methods. Although initial exo III studies had suggested multiple (up to 9) sites of protein-binding to the ftz promoter, more recent gel mobility shift analyses indicate that only 3 major protein-DNA complexes are observed using this assay, although each complex may possess multiple exo III protections. One factor, NF-ftz1, has been purified to homogeneity from several kilograms of Drosophila embryos. Antibodies to NF-ftz1 have been generated that react with both the native and denatured protein, and are being utilized to screen a lambda gt11 expression library. The purification of a second factor identified above will commence shortly.

Publications:

Zimarino V, Wu C. Induction of sequence-specific binding of Drosophila heat shock activator protein without protein synthesis, Nature 1987;327:727-30.

Krawczyk Z, Wu C. Isolation of RNA for dot hybridization by heparin-DNaseI treatment of whole cell lysate, Anal Biochem 1987;165:20-7.

Wu C, Wilson S, Walker B, Dawid I, Paisley T, Zimarino V, Ueda H. Purification and properties of Drosophila heat shock activator protein, Science 1987;238:1247-53.

Wu C, Tsai C, Wilson S. DNA-affinity chromatography. In: Setlow J, ed. Genetic Engineering, vol 10; in press.

Wu C. Analysis of DNaseI hypersensitive sites in chromatin. In: Kornberg RD, Wasserman P, eds. Methods in Enzymol; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05264-07 LB

PERIOD COVERED
October 1, 1987 to September 30, 1988

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	Research Chemist	LB NCI
Edward 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 20892

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.)*

Studies on the structure and function of type II intracisternal A-particle (IAP) retroviral elements have continued. To define factors involved in IAP element transposition, we have studied the MOPC-315 and MOPC-104E myeloma cell lines in which the type IIB subclass of elements has undergone a marked amplification. Type IIB elements also encode the major IAP transcripts in these cells. We have found endonuclease activity in IAP fractions from both myelomas as demonstrated by conversion of supercoiled plasmid DNA to relaxed and linear forms. In addition, DNA molecules consistent in structure with a linear type IIB IAP provirus were present in the cytoplasm of MOPC-315 cells.

Rabbit antisera to an oligopeptide predicted from the nucleotide sequence of the endonuclease gene in a full length genomic IAP element reacted with MOPC-315 cells when assayed by immunofluorescence and with a protein in cell fractions on Western blots. The antisera also reacted with a fusion protein produced in bacteria transfected with a randomly selected genomic type IIB IAP element, demonstrating that the endonuclease reading frame was open in this otherwise defective endogenous provirus.

Sequencing has revealed that the type II insertions introduce a splice acceptor site not present in a full size element. The splicing mechanism may result in higher levels of endonuclease than can be achieved from expression of elements in which a frame shift is required for translation of the RNA, and subsequent cleavage of the polymerase gene product has to occur.

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

Project Description

Objectives:

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.

Major Findings:

A. Endonuclease (Integrase Function)

To define some of the factors involved in IAP element transposition, we have studied the MOPC-315 and MOPC-104E myeloma cell lines in which the type IIB subclass of elements has undergone a marked amplification compared with embryonic cells and other myelomas. Type IIB elements are also the source of the major transcripts in these cells. These elements contain an extensive deletion encompassing the gag and polymerase genes, but have an intact endonuclease coding region. We have found endonuclease activity in IAP fractions from both myelomas as demonstrated by conversion of supercoiled plasmid DNA to relaxed and linear forms. The activity is tightly bound to the IAPs in the presence of non-ionic detergent up to 1% and does not require ATP.

Rabbit antisera were raised to an oligopeptide predicted from the nucleotide sequence of the endonuclease gene in a full length genomic IAP element. These antisera were used to test whether the type IIB elements are capable of encoding an endonuclease function. A type IIB element from mouse embryo DNA was cloned into a bacterial expression vector in which production of the endonuclease would occur as a fusion protein with trpE. This construct produced high levels of a 68 KDa protein (trpE codes for a protein of 37 KDa) which reacted with the endonuclease antibody, indicating that a randomly selected type IIB element had an open reading frame for endonuclease. The fusion protein has been isolated and used to immunize a rabbit to produce antibodies to the whole endonuclease. The rabbit antibodies to the endonuclease peptide detected a protein in whole cells when assayed by immunofluorescence and in cell fractions assayed by Western blots. Both of these reactions were blocked by preincubation of the serum with peptide. The reaction of the cells was relatively strong compared with that of the fractionated proteins on the Western blot, suggesting some loss of activity associated with the denaturing conditions required for blotting.

B. Provirus

We have found that MOPC-315 myeloma cells contain linear type IIB proviral elements in the cytoplasm, presumably as precursors for integration. When cells were lysed in the absence of detergent and DNA was extracted from the membrane fraction (10K pellet) and analyzed on a Southern blot without restriction endonuclease digestion, a discrete 4 Kb band corresponding in

size to that expected for a linear type IIB element was detected by hybridization of the subsequent blot with an IAP element probe. If cells were lysed in the presence of detergent, this material was released from the 10K pellet, but remained particulate and was sedimentable at 40K.

C. cDNA Library from MOPC-315 Cells

A cDNA library derived from MOPC-315 poly(A)+ RNA was cloned in lambda phage gt11 to permit isolation of clones which express protein. One clone, isolated by virtue of reaction with antibody to the IAP structural protein p73, was too short to permit further characterization. Many of the cDNAs were partial and non-overlapping. None of the clones reacted with antibody to endonuclease. Two of the longer, overlapping cDNAs have been sequenced and compared to a genomic type IIB clone and a full size type I IAP clone, MIA14. The precise structure of the IIB element has been defined. Comparison of the common sequences shows that this element and the two cDNAs are more closely related to one another than they are to the genomic type I element. Sequencing of these elements has revealed that the type II element specific insertion introduces a splice acceptor site. Splicing of an RNA from the 5' IAP splice donor site to this site might result in higher levels of endonuclease than can be achieved from expression of full-sized elements in which a frame shift is required for translation of the RNA and subsequent cleavage of the polymerase product has to occur.

Publications:

Mietz JA, Grossman Z, Lueders KK, Kuff EL. Nucleotide sequence of a complete intracisternal A-particle genome: relationship to known aspects of particle assembly and function. J Virol 1987;61:3020-9.

Kuff EL, Lueders KK. The intracisternal A-particle gene family: structure and functional aspects, Adv Cancer Res 1988;51:183-276.

Kuff EL, Lueders, KK. Structure and evolution of retroviruses with homology to the transposable intracisternal A-particle elements of mice. In: Kingsman AJ, Chater KF, Kingsman SM, eds. Transposition. Cambridge: University Press, 1988;247:70.

Lueders KK, Kuff EL. Transposition of intracisternal A-particle genes, Prog Nucleic Acid Res Mol Biol 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05265-06 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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	Chemist	LB	NCI
N. D. Vu	Chemist	LB	NCI
A. Carroll	Biologist	LB	NCI
H. Foster	Lab Technician	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Protein Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

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 20,000-Da subunits of vertebrate nonmuscle myosins are phosphorylated by specific calcium-calmodulin dependent kinases, myosin light chain kinases. This phosphorylation regulates the actin-activated ATPase activities of these myosins and appears to play a central role in the regulation of cellular motile activities. However, there is disagreement as to the mechanism of this regulation. Other investigators have reported that phosphorylation causes large increases in the actin-activated ATPase activities of these myosins and have hypothesized that this phosphorylation acts as an on/off switch. However, we have found that at high actin concentrations or in the presence of tropomyosin, the ATPase activity of filamentous cell thymus myosin is almost independent of phosphorylation. In an attempt to reconcile the difference between our results and those of other investigators, we have determined the actin-activated ATPase activity and filament stability of thymus myosin under a variety of conditions. Both the unphosphorylated and phosphorylated myosins needed to be filamentous for their ATPase activities to be activated by actin. Under conditions where phosphorylation increased myosin filament formation, there were large increases in actin-activated ATPase activity upon phosphorylation, but under conditions where phosphorylation had only a small effect on myosin filament formation it had only a small effect on actin-activated ATPase activity. These results suggest that phosphorylation may regulate actomyosin based motile activities in vertebrate nonmuscle cells by regulating myosin filament assembly. As cytoplasmic myosins are also phosphorylated in vivo by the calcium-phospholipid dependent protein kinase, protein kinase C, we have examined the effect of protein kinase C phosphorylation on the actin-activated ATPase activity and filament assembly of thymus myosin.

Project Description

Objectives:

Our general goals are to understand the roles of cytoplasmic 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.

Major Findings:

Vertebrate smooth muscle and nonmuscle myosins have similar properties and subunit compositions being composed of two 200,000-Da heavy chains and two pairs of light chains, M_r 17,000 and 20,000. The 20,000-Da light chains, LC20, are phosphorylated by specific calcium-calmodulin dependent kinases (myosin light chain kinases) and dephosphorylated by calcium insensitive phosphatases. This phosphorylation is thought to play a central role in the regulation of smooth muscle contraction and nonmuscle contractility. In vitro this phosphorylation affects both the actin-activated ATPase activities of these myosins and their assembly into filaments. We have previously shown that the actin-activated ATPase activities of filamentous, unphosphorylated and phosphorylated calf thymus myosins have the same maximum rates, V_{max}. Phosphorylation regulates the actin-activated ATPase activity of this cytoplasmic myosin by increasing its apparent affinity for actin. However, at high actin concentrations or in the presence of tropomyosin, the ATPase activity of thymus myosin is almost independent of phosphorylation. The observation that the actin-activated ATPase activities of unphosphorylated and phosphorylated myosins have the same V_{max} was obtained under conditions in which both the unphosphorylated and phosphorylated myosins were greater than 90% filamentous. These conditions were chosen to avoid the complication of comparing the actin-activated ATPase activities of filamentous and monomeric myosins. When the assays were performed at a higher ionic strength, the ATPase activity of the unphosphorylated myosin even at very high tropomyosin-actin concentrations was less than 1/3 that of the phosphorylated myosin. Since under similar conditions phosphorylation has been shown to stabilize thymus myosin filaments, this difference in ATPase activity may result from the different states of myosin assembly under these conditions. This suggests that phosphorylation might regulate the actin-activated ATPase activity of thymus myosin by regulating its assembly into filaments. We have examined this possibility by determining the actin-activated ATPase activity and filament assembly of thymus myosin under a variety of conditions.

Thymus myosin filament assembly and actin-activated activity were affected by ionic strength, Mg^{2+} concentration, myosin concentration, and LC20 phosphorylation. Phosphorylated myosin remained filamentous at higher KCl and lower Mg^{2+} concentrations than did the unphosphorylated myosin. When the unphosphorylated and phosphorylated myosins were filamentous their ATPase activities were activated 10-20 fold by actin, but when they were monomeric their ATPase activities were not activated or only very slightly activated by actin. Under conditions in which LC20 phosphorylation caused a large increase in myosin filament assembly, it caused a large increase in actin-activated ATPase activity, but

under conditions in which phosphorylation had only a small effect on myosin filament assembly, it had only a small effect on actin-activated ATPase activity. By varying the myosin concentration, it was possible to have under the same assay conditions mostly monomeric myosin or mostly filamentous myosin; the actin-activated ATPase activities of the filamentous myosins were much greater than those of the monomeric myosins. Thus, the state of myosin assembly appears to be of primary importance in determining the actin-activated ATPase activity of thymus myosin. This conclusion is very different from that obtained with gizzard smooth muscle heavy meromyosin. Since gizzard heavy meromyosin cannot form filaments, regulation of its actin-activated ATPase activity by LC20 phosphorylation must be independent of filament assembly. This result with gizzard heavy meromyosin has been used by others to infer that the regulation of the actin-activated ATPase activity of nonmuscle myosins by light chain phosphorylation is also independent of filament assembly, i.e. the V_{max} of the actin-activated ATPase activity of a filamentous, unphosphorylated nonmuscle myosin would be much less than that of the filamentous, phosphorylated myosin. However, they did not determine the actin-activated ATPase activities unphosphorylated and phosphorylated nonmuscle myosins under conditions in which both types of myosin are filamentous.

If phosphorylation regulates myosin filament assembly in vivo, it could be an effective system for regulating actomyosin based motile activities in nonmuscle cells. In the unstimulated cell, the unphosphorylated myosin would be monomeric and free to diffuse through the cell. Upon stimulation, a rise in internal Ca^{2+} concentration activates myosin light chain kinase which then phosphorylates myosin. The phosphorylated myosin forms filaments which interact with actin to cause movement. However, if the unphosphorylated myosin in vivo is filamentous, the cell must have some other mechanism to regulate its interaction with actin. The state of myosin assembly in nonmuscle cells is unknown.

Stimulation of platelets by thrombin and basophils by antigen results in an increase in myosin LC20 phosphorylation. Recent experiments have shown that part of the LC20 phosphorylation in platelets and most of it in the basophils results from phosphorylation by protein kinase C, the calcium-phospholipid dependent protein kinase. Based on experiments with gizzard heavy meromyosin, it has been suggested that this phosphorylation inhibits the interaction of cytoplasmic myosins with actin. Our preliminary experiments indicate that protein kinase C phosphorylation of thymus myosin has little if any effect on its interaction with actin.

Publications:

Wagner PD, Vu ND. Actin-activation of unphosphorylated gizzard myosin, J Biol Chem 1987;262:15556-62.

Vu N-D, Wagner PD. Effects of proteolysis on the ATPase activities of thymus myosin, Biochemistry 1987;26:4847-53.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05267-04 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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 & Genetics Section LB NCI

D. Chattoraj	Microbiologist	LB NCI	E. Maguin	Visiting Fellow	LB NCI
K. Tilly	Sr. Staff Fellow	LB NCI	S. Pal	Visiting Fellow	LB NCI
S. Jafri	Guest Researcher	LB NCI	S. Dasgupta	Visiting Scientist	LB NCI
K. Muraiso	Guest Researcher	LB NCI	N. Grover	Visiting Scientist	LB NCI
K. Breiter	Technician	LB NCI	S. Dasgupta	Visiting Scientist	LB NCI
A. Ghosh	Visiting Scientist	LB NCI			
B. Funnell	Visiting Associate/Visiting Scientist	LB NCI			

COOPERATING UNITS *(if any)*

Dr. S. Wickner, LMB, NCI; Dr. N. Trun, LMB, NCI

Dr. E.B. Hansen, Danish Technical University, Denmark

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Developmental Biochemistry and Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.5	8.0	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.)*

We continue our studies of the replication and partition mechanisms that account for the stable inheritance of unit-copy bacterial plasmids for which P1 plasmid prophage serves as exemplar. In the period of this report we have (1) provided more compelling evidence than had been previously obtained for a DNA looping mediated by the P1-specified initiator protein, RepA, (2) shown, by site-directed mutagenesis and deletion analysis, that RepA protein appears not to be the cause of the replication inhibition observed when the repA gene is overexpressed, but that mutations within repA appear able to relieve this inhibition. These mutations are being exploited in the construction of a versatile miniP1-based cloning and expression vector, (3) devised promising selection procedures for the isolation of host mutants affecting plasmid maintenance, (4) determined the contribution of the recently characterized lytic P1 replicon to plasmid replication as a function of the expression of phage immunity genes, (5) demonstrated that P1 is an addictive genetic element; once acquired, the plasmid becomes necessary for continued bacterial survival, (6) shown that one of the two P1-determined proteins that are essential for partitioning of the plasmid to daughter cells binds to the plasmid centromere with an integration host factor (IHF). IHF is the first host component of a plasmid partitioning system to have been identified.

GPO 914-918

Project Description

Objectives:

We aim to elucidate mechanisms that (1) precisely control the initiation of DNA replication, (2) assure the stable maintenance of replicons by (a) appropriately partitioning them to daughter cells and (b) assuring the death of cells from which the replicon has been lost.

Major Findings:

A. DNA Looping Revisited

Experiments previously reported provided evidence for a role of DNA looping in the control of P1 plasmid replication. The evidence was based, in part, on electron microcopy of DNA loops mediated by the P1 initiator protein, RepA, in which the relevant regions of a miniP1 replicon were brought together and then fixed with glutaraldehyde. A modified procedure devised by Drs. Pal and Chattoraj eliminates the possibility that the looping observed was an artifact of glutaraldehyde cross-linking. In addition to increasing confidence in the significance of the original observation, this result provides an assay for screening prospective looping-negative repA mutants.

B. Extension of the Range of repA Expression

RepA-dependent replication is inhibited by repA overexpression. Site-directed mutagenesis of the repA region has provided Drs. Muraiso and Chattoraj with preliminary evidence as to the nature of the inhibitor. Mutations in repA that increase copy number when repA is overexpressed were obtained and analyzed by Drs. Pal and Chattoraj and are being used by Drs. Muraiso and Chattoraj in the construction of a versatile cloning and expression vector.

C. Demonstration that P1 Plasmid is Addictive

P1 plasmid loss was found by Dr. Yarmolinsky and Ms. Jafri to cause cellular filamentation and death. From a λ library of P1 fragments, chimeras that prevent this lethality and that presumably determine the synthesis of an unstable gene product whose function is to antagonize a relatively stable P1-specified lethal agent were isolated. A collaboration between Dr. Nancy Trun (Laboratory of Molecular Biology) and Dr. Yarmolinsky on the nature of this instability is being initiated.

D. Host Participation in Plasmid Maintenance

Dr. Ghosh has developed a scheme to isolate bacterial mutants that selectively reduce P1 replication, potentially helpful tools in understanding the controls that assure the maintenance of a low copy number plasmid.

In order to identify bacterial genes involved in P1 plasmid partitioning, Dr. Tilly has developed two methods for isolating E. coli mutants in which

the stability of P1 is altered, of which the second method is selective for partitioning mutants. So far, several strains with mutations that selectively affect the replication of miniP1 plasmids have been isolated.

E. Simulations of Alternative Models of Partitioning

The consequences of various models of plasmid partitioning on the kinetics of appearance of cured cells are being examined by computer simulation. Two kinds of chimeras composed of well-understood components are being considered for this purpose: P1*par*-*oriC*(*E. coli*) and P1*par*-miniR1. Sensitivity can be increased by selecting the cells in the original sample to be all at a predetermined age by using the membrane elution method. Simulations are by Dr. Grover. Experimental work may involve a collaboration between members of this laboratory and Dr. Charles Helmstetter (Roswell Park, New York).

F. Participation of Integration Host Factor in P1 Plasmid Partitioning

Dr. Funnell has shown that one of two P1-determined proteins that are essential for partitioning of the plasmid binds to the plasmid centromere, *parS*. Fractionation of extracts from *E. coli* cells overproducing this protein (ParB) and studies with mutant hosts revealed that integration host factor (IHF), in addition to purified ParB protein, is required to obtain a maximal binding to *parS*. Binding studies *in vitro*, and competition experiments *in vivo* suggest that two types of ParB protein-*parS* DNA complexes can exist that differ in (1) the presence of IHF, (2) the amount of *parS* sequence with which the proteins interact, and (3) the specificity of their participation in partition. IHF is the first host component of a plasmid partitioning system to have been identified.

Publications:

Swack JA, Pal SK, Mason RJ, Abeles A, Chattoraj DK. P1 plasmid replication: measurement of initiator protein concentration *in vivo*, J Bacteriol 1987;169: 3737-42.

Wickner SH, Chattoraj DK. Replication of mini-P1 plasmid DNA *in vitro* requires two initiation proteins: the products of P1 *repA* and *E. coli dnaA*, Proc Natl Acad Sci USA 1987;84:3668-72.

Yarmolinsky MB. Bacteriophage P1. In: O'Brien SJ, ed.: Genetic Maps. New York: Cold Spring Harbor Laboratory, 1987;4:38-47.

Funnell BE. Mini-P1 plasmid partitioning: excess *parB* protein destabilizes plasmids containing the centromere, *parS*, J Bacteriol 1988;170:954-60.

Chattoraj DK, Mason RJ, Wickner SH. Mini-P1 plasmid replication: the autoregulation-sequestration paradox, Cell 1988;52:551-7.

Yarmolinsky MB. The legacy of Luigi Gorini, Trends Biochem Sci 1988;13:77-8.

Hemmingsen SM, Woolford C, Vander Vies SM, Tilly K, Dennis DT, Georgopoulos CP, Hendris RW, and Ellis RJ. Homologous plant and bacterial proteins chaperone oligomeric protein assembly, Nature 1988;333:330-4.

Yarmolinsky MB, Sternberg N. Bacteriophage P1. In: Calendar R, ed. The bacteriophages, vol 1. New York: Plenum Press; in press.

Funnell BE. Participation of Escherichia coli integration host factor in the P1 plasmid partition system, 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 05268-01 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Mechanisms of Meiotic Recombination

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

Michael Lichten	Senior Staff Fellow	LB	NCI
Carol Wu	Chemist	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Developmental Biochemistry and Genetics

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

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.)*

During the period of this report, we have initiated research aimed at understanding the molecular mechanism of meiotic recombination in the yeast Saccharomyces cerevisiae and the means by which yeast exerts control over meiotic recombination. We have developed techniques to detect heteroduplex DNA molecules formed during recombination and used these techniques to determine the specificity of base-pair mismatch correction and the polarity of strand transfer during meiosis. We have also created yeast strains and developed techniques to carry out synchronous meiosis in mass culture and to isolate chromatin from cells undergoing meiosis. We are using these techniques to determine if the meiotic transcriptional activity and chromatin structure of various regions correlate with their level of meiotic recombination. Finally, we have completed an examination of position effects in mitotic recombination.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

Our immediate objective is an understanding of the molecular mechanism of meiotic recombination, using the yeast Saccharomyces cerevisiae as a model system. We have developed a methodology which allows detection of heteroduplex DNA products of recombination, and are applying this methodology to a detailed examination of the molecular exchange and mismatch correction events which occur during meiosis.

Using meiotic recombination as a starting point, we intend to examine the means by which control is exerted over the level of meiotic recombination in different parts of the genome and the gene products which participate in that control. We are completing an investigation of the possible roles that transcription and chromatin structure have in regulating the level of meiotic recombination at different genomic locations.

Major Techniques Employed and Major Findings:

A. Detection of Heteroduplex DNA Products of Meiotic Recombination

Michael Lichten has adapted the denaturant-gradient gel technique of Fischer and Lerman (Proc. Natl. Acad. Sci. USA 80:1579) to allow detection of heteroduplex DNA molecules containing single base-pair mismatches. This modification not only allows the sensitive detection of such molecules, but also allows resolution of the two mismatch configurations possible at a given site. As a first step, we have applied this methodology to examination of the meiotic behavior of a G > C transversion mutation in the initial AUG codon of the yeast ARG4 gene. We have determined that, of the two possible base-pair mismatches (G/G and C/C), only one (C/C) escapes mismatch correction to a significant extent and survives to be found in spores. Thus, at least with regard to these two mismatches, yeast follows the same mismatch-correction rules as do E. coli and S. pneumoniae. From a combination of these physical studies and genetic studies by Drs. D. Treco, A. Nicholas and J. Szostak at Harvard Medical School, we have been able to deduce which DNA strand of the arg4 gene is transferred to form heteroduplex DNA during meiotic recombination, and find that it is predominantly the coding strand. We are currently extending this analysis to other mutations in the arg4 gene and to other yeast genes, to confirm the correctness and generality of these findings.

B. Analysis of Transcription and Chromatin Structure During Meiosis

On a large number of organisms, from yeast to man, different regions of the genome display widely different frequencies of meiotic recombination. It has recently been suggested that, in yeast, this is a general property of a region, since a reporter gene inserted in a "cold" region displays a low frequency of meiotic recombination, while the same gene inserted in a "hot" region displays a high frequency of meiotic recombination (Lichten et al., Genetics 115: 233). Carol Wu and Michael Lichten have taken

advantage of this finding to search for properties of regions displayed during meiosis which correlate with recombination activity. Preliminary findings indicate that both "hot" and "cold" copies of a reporter gene display the same level of transcription during meiosis. These findings appear to rule out transcription as a primary determinant of the level of recombination in a gene. We are now extending this study to an examination of alterations in chromatin structure of the same reporter gene inserted in various regions of the genome which may occur during meiosis. The strains and techniques developed during these studies (synchronous sporulation in mass culture, chromatin isolation from cells undergoing meiosis) will form an essential foundation for later studies, which will involve a real-time examination of meiotic recombination and mismatch correction.

C. Lack of Position Effects in Mitotic Recombination

Previous studies (Lichten et al., cited above) have indicated that the different meiotic recombination activities of different regions are reflected in the level of meiotic recombination in a reporter gene inserted in those regions. Michael Lichten has used the same approach to determine the rate of mitotic recombination in several regions of the yeast genome. The rate of mitotic recombination was found to be roughly the same in all regions examined, even though these regions differed by as much as 40-fold in their level of meiotic recombination. It therefore appears that the observed wide range in levels of meiotic recombination in different regions of the genome is meiosis-specific and may reflect basic changes, induced during meiosis, in either chromosome structure or the nature of the recombination machinery.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 05269-01 LB
Formerly part of
Z01 CB 05214-17 LB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
DNA Synthesis in Mammalian Cells: Studies of Nucleic Acid Binding Proteins

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

S. H. Wilson Medical Officer LB NCI

COOPERATING UNITS *(if any)*
H.R. Guy, NCI; K. Seamon, FDA; K. Williams, Yale; R. Karpel, University of Maryland; F. Cobianchi, CNR, Pavia, Italy; A. Steinberg, NIDDKD

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Nucleic Acid Enzymology Section,

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	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.)*

Physical biochemical studies of a single-stranded nucleic acid binding protein termed A1 were conducted. The protein binds cooperatively to either RNA or DNA and the full length protein binds much tighter than a truncated A1 protein lacking the glycine-rich COOH-terminal domain (residues 185-319). Our studies suggest the mechanism of A1 binding is similar to that of two well prokaryotic ssDNA binding proteins in that binding involves close approach of aromatic amino acids with nucleotide bases. We found that both the NH_2-terminal and COOH-terminal domains make significant contributions to the overall free energy of binding of A1 to nucleic acids.

Project Description

Objectives:

The ultimate objective of this research program is to understand mechanisms of DNA synthesis in mammalian cells. Our main approaches are enzymology of DNA synthesis proteins and examination of DNA replication *in vitro* using purified polymerases and other required proteins.

It is anticipated that these studies on the properties, specificities, and regulation of DNA replication proteins will, in conjunction with results from other laboratories, help answer important questions about mechanisms and control of DNA replication in the mammalian cell.

Major Findings:

A. Structure-Function Studies of the Single-Stranded Nucleic Acid Binding Protein: Mammalian hnRNP Protein A1

Immediately following transcription, heterogeneous nuclear RNA (hnRNP) becomes associated with a group of proteins to form a ribonucleoprotein (hnRNP) complex that is believed to function in the processing, stabilization and transport of unique sequence transcripts. Earlier, we cloned a cDNA for the A1 hnRNP protein and over-produced the protein in *E. coli*. We now are using a variety of physiochemical approaches to identify functional domains in the protein. Sequencing revealed that the first 184 amino acids in A1 contain an internal repeat such that when residues 3-93 are aligned with 94-184, 33% of the amino acids are identical. Limited proteolysis and photochemical cross-linking experiments indicate that the internal repeats in A1 correspond with two independent nucleic acid binding domains. With a view toward understanding structure-function relationships of a protein component of the eukaryotic heterogeneous ribonucleoprotein complex, we have studied the interactions of recombinant A1 protein and its N-terminal two-thirds portion (UP1) and C-terminal domain with the fluorescent polynucleotide, poly(ethenoadenylic acid), poly(εA). The overall binding affinity of A1 for poly(εA) is significantly greater than that of UP1. This can be partly, but not entirely explained by the existence of cooperativity in A1-nucleic acid interactions: the cooperativity parameter, ω, for A1-poly(εA) interaction is about 30, whereas UP1 binds nucleic acids non-cooperatively (ω=1). Gel retardation experiments performed with M13 DNA also show cooperative binding for whole A1, and non-cooperative interaction for UP1. Analogous experiments with the C-terminal domain show that this polypeptide is capable of cooperative binding to single-stranded nucleic acids. These results suggest that A1 possesses at least two potential binding sites for nucleic acids: one within N-terminal UP1, one within the C-terminal portion. Both whole A1 and the C-terminal domain seem capable of protein-protein association. A synthetic peptide that is a 6-8mer of the consensus sequence of the A1 C-terminus, GNFGGGRGGNYGGSRG (~10,000 daltons) binds poly(εA) tenaciously and cooperatively (ω~25). Competition titrations run in the presence of other polynucleotides, e.g. poly(U), indicate that this polypeptide has a strong affinity for other nucleic acids, as well.

Experiments are in progress to test the contributions of specific amino acids to the binding affinity via systematic variation of the sequence.

In collaboration with A. Steinberg and colleagues, we found that patients with autoimmune diseases have circulating antibodies to the A1 protein.

Publications:

Cobianchi F, Karpel RL, Williams KL, Notario V, Wilson SH. Mammalian hnRNP complex protein A1: large-scale overproduction in E. coli and cooperative binding to single-stranded nucleic acids, J Biol Chem 1988;263:1063-71.

Merrill BM, Stone KL, Cobianchi F, Wilson SH, Williams KR. Phenylalanines that are conserved among several RNA-binding proteins form part of a nucleic acid-binding pocket in the A1 hnRNP protein, J Biol Chem 1988;263:3307-13.

Jensen L, Kuff EL, Wilson SH, Steinberg A, Klinman D. Antibodies from patients and mice with autoimmune diseases react with recombinant hnRNP core protein A1, Autoimmunity; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05270-01 LB
Formerly part of
Z01 CB 05214-17 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

DNA Synthesis in Mammalian Cells: Mechanism of HIV Reverse Transcriptase

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

S. H. Wilson	Medical Officer	LB	NCI	
J. Abbotts	IRTA Fellow	LB	NCI	
C. Majumdar	Guest Researcher	LB	NCI	(6 months)

COOPERATING UNITS *(if any)*

G. Zon and K. Seamon, FDA; S. Broder and J. Cohen, NCI

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.7	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 continued biochemical studies of mammalian DNA replication proteins. Steady-state kinetic analysis of the HIV reverse transcriptase revealed an over-all kinetic scheme for reverse transcriptase mechanism. A DNA segment containing the coding region for this enzyme was subcloned in an expression vector and the enzyme was overproduced in E. coli. This recombinant enzyme is under study.

The model DNA polymerase, Pol I l.f., forms DNA in a processive fashion, remaining associated with the template for many dNMP additions. However, after any given dNMP addition along a template some enzyme molecules terminate synthesis and fall off the template. This termination is quantified by analysis of reaction product size using a sequencing gel. We found that with a natural DNA (ØX174) template·primer system, certain points along the single-stranded DNA template are strong termination sites for Pol I l.f. The mechanism of this termination was studied. Observations were extended with a second defined template, containing one inosine residue in an otherwise $d(T)_n$ homopolymer. Termination at the I residue is modulated by dCTP and by other modifications in the reaction conditions. The results are consistent with a model in which termination occurs with several enzyme forms that are in equilibrium in an ordered catalytic mechanism.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

The ultimate objective of this research program is to understand mechanisms of DNA synthesis in mammalian cells. Our main approaches are enzymology of DNA synthesis proteins and examination of DNA replication in vitro using purified polymerases and other required proteins.

It is anticipated that these studies on the properties, specificities, and regulation of DNA replication proteins will, in conjunction with results from other laboratories, help answer important questions about mechanisms and control of DNA replication in the mammalian cell.

Major Findings:

Structure-Function Relationships of DNA Polymerases, E. coli DNA Polymerase I Large Fragment (Pol I l.f.) and HIV Reverse Transcriptase

Termination mechanism

The model DNA polymerase, Pol I l.f., forms DNA in a processive fashion, remaining associated with the template for many dNMP additions. However, after any given dNMP addition along a template some enzyme molecules terminate synthesis and fall off the template. This termination is quantified by analysis of reaction product size using a sequencing gel. With a natural DNA (ØX174) template•primer system, certain points in single-stranded DNA are strong termination sites for Pol I l.f. Termination is higher where the incoming nucleotide is a pyrimidine. This also was observed with synthetic oligonucleotide templates, indicating that termination could be examined with selected modifications of individual template residues. Observations with the ØX174 DNA template system thus were extended with a defined template containing one inosine residue in an otherwise $d(T)_n$ homopolymer. Termination at the I residue is modulated by dCTP and decreases as dCTP concentration increases. A similar relationship is seen with dCTPαS, but termination is higher at given concentrations of this thiol derivative than with dCTP. Pyrophosphate decreases general processivity in this system, but does not counteract the effect of increasing dCTP. Hill plot analysis of the dCTP effect in the inosine-containing template system gave a linear plot with Hill coefficient of 0.34, suggesting that dCTP influences termination at several steps in the polymerase reaction scheme. Substituting a methylated template base for I also increased termination, producing essentially absolute blocks to processive synthesis. The results are consistent with a model in which termination occurs with several enzyme forms that are in equilibrium in an ordered catalytic mechanism.

A study of steady-state kinetics of polymerization by purified HIV DNA polymerase (reverse transcriptase) has been conducted. DNA synthesis was examined using a system of poly r(A) as template, oligo d(T) as primer, and dTTP as nucleotide substrate. The substrate initial velocity patterns point to an ordered mechanism with template primer adding first. Product inhibition kinetics with either pyrophosphate or phosphonoformate are consistent

with this mechanism. The HIV reverse transcriptase acts processively in this replication system, but exhibits some probability of terminating after each dTMP addition to the nascent chain. The probability of terminating was ~20-fold higher after the first dTMP addition than after subsequent additions. With this information on the mode of polymerization, appropriate kinetic models and steady-state rate equations are discussed. In further studies, we found that a heterologous polynucleotide, poly (rC), is a potent inhibitor of the enzyme. The pattern of this inhibition is uncompetitive against template•primer, suggesting that interaction with free enzyme is not the mechanism of the inhibition.

In further studies we found that the free enzyme interacts with the primer portion of the template•primer complex. This was extended to the use of phosphorothioate oligonucleotides as enzyme inhibitors and as primers. The phosphorothioate derivatives have much higher affinity for the enzyme than the oxygen homologs. Finally, the coding region for the HIV reverse transcriptase was excised from a proviral DNA clone and then subcloned in the E. coli expression vector pKK223. The manufactured enzyme is now under investigation.

Publications:

Majumdar C, Abbotts J, Roder S, Wilson SH. Studies on the mechanism of HIV DNA polymerase: steady-state kinetics processivity and polynucleotide inhibition, J Biol Chem; in press.

Abbotts J, SenGupta DN, Zon G, Wilson SH. Studies on the mechanism of Escherichia coli DNA polymerase I large fragment: effect of template sequence and substrate variation on termination, J Biol Chem; in press.

LABORATORY OF MATHEMATICAL BIOLOGY

SUMMARY

October 1, 1987 through September 30, 1988

Research in the Laboratory of Mathematical Biology (LTB) is grouped in several broad basic areas: macromolecular structure and function, membrane structure and function, immunology, pharmacokinetics, and computational and modeling methodologies. The work is both theoretical and experimental. Application of the basic understanding of these biological systems, serving as models for aspects of the cancer process, is accomplished through the use of advanced computing. Close collaborations provide valuable feedback and knowledge transfer between research domains. The Laboratory often develops computational and experimental methodology that is utilized by researchers of the entire biomedical community. Many of the theoretical studies have only been possible through the use of new computer facilities at the Advanced Scientific Computing Laboratory, FCRF.

Sequence Analyses in Virology, Cell and Molecular Biology. In the Office of the Chief computerized analyses are used extensively along with experimental techniques of biochemistry, virology, and electron microscopy to study picornaviruses, adenoviruses and other virus-cell systems.

The availability of a large number of nucleotide and amino acid sequences enables detailed studies of a particular system as well as searches for general trends. Detailed studies can involve structural computations and the effect of (single point) mutations. Searches for general trends may involve comparisons of structures of related genes. General patterns can also be discerned by studies of sequences fulfilling analogous functions and taken from a variety of genes/organisms (e.g. promoters) or by searches for overall sequence characteristics (e.g. as required by genome packaging)(S. Le, K. Currey, A. Konopka, R. Nussinov, J.V. Maizel and J. Owens).

Picornaviruses cause diseases typified by polio, colds, hepatitis, and foot-and-mouth diseases. Sequences of these viruses have been examined for relationships within the family and to other known and hypothetical proteins. Secondary structures of the RNA's have been found to vary with respect to pathological and sequence variations. A single base change at position 472 in Sabin polio type 3 correlates with reversion to neurovirulence; it also changes the predicted stem and loop secondary structure (Maizel, 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 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 (Chatterjee, Maizel, and Owens).

New analytical tools for studies of proteins and nucleic acids have been developed and implemented (Nussinov, Maizel and Owens). Numerical methods aid in the prediction of secondary structures, splice sites, promoters, and recombination sites in nucleic acids. Graphic representations reveal homology, and reverse complementarity. These programs were developed and have been installed on a variety of computer systems at the Advanced Scientific Computing Laboratory. Structures up to 2000 bases in size have been predicted. Methods to assess the significance of predictions have used Monte Carlo simulations, evolutionary comparisons and biochemical data. Also protein secondary structure has been predicted from amino acid sequences. New sequences were 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.

There is work on the identification and characterization of T cell antigenic sites (Cornette, Margalit, Spouge, and Berzofsky). An algorithm to identify potential T cell sites based on helical amphipathicity and other characteristics is used to predict likely antigens. Some of the first peptides predicted this way have been tested in the laboratory of Jay A. Berzofsky and have proven to be antigenic.

An examination of DNA mapping strategies has been initiated in order to identify optimal strategies to map chromosomal DNA of $\simeq 10^8$ base pairs (Cornette and DeLisi). The overall goal of the mapping is to generate a library of clones of DNA fragments that covers the entire DNA and to obtain partial but sufficient information about the fragments in order to determine their order in the original DNA. The proposed schemes are tested by Monte Carlo and other probabilistic methods on randomly generated DNA.

Studies on the primary structure of beta 1-4 galactosyltransferase (1-4 galtransferase) are continuing (Qasba, Masibay, and A. Puri). The results of these studies show 1-4 galtransferase to be synthesized as a larger precursor protein of 401 residues including an NH_2-terminal 72 residue peptide with the membrane anchoring domain as well as the potential protease susceptible sequence ARG-X-X-ARG-ARG-LEU that is missing from the secreted and soluble form of the enzyme. The remaining 320 residue protein carries the catalytic domain and is the soluble form of the protein. The topology of the Golgi and cell surface 1-4 galtransferase is predicted to be similar to a class of membrane proteins which have an NH_2 terminal "stem" structure extending from the luminal side of the transmembrane bilayer to the cytoplasmic side. The bulk of the protein structure, which carries the main function, faces the lumen side. For the structure-function studies, the cDNA has been inserted into a modified baculovirus vector which is being used for the production of normal and altered proteins in baculovirus infected insect cells or in frog oocytes by injecting the cDNA specific mRNA transcribed in vitro by T7 polymerase. The cDNA sequences of 1-4-galtransferase and alpha-lactalbumin, are engineered in such a way that the cDNA sequences have the potential to produce either soluble and secreted forms or membrane anchored forms of the protein. Also for detailed

structure-function relationship studies various cell lines are being tested for the activity of 1-4 galtransferase, the molecular form of the protein produced, the mRNA levels for the enzyme and the mRNA size class produced. Such cell lines will be used for transfection experiments to determine the structure-function relationship of galtransferase and alpha-lactalbumin.

Theoretical Molecular Structure.In the laboratory we are studying biological macromolecules and their properties, including studies on peptides, proteins, DNA and RNA.

One of the principal difficulties in achieving the correct folded conformation of a protein is the overwhelmingly large number of possible conformations. Restricting the space in which the conformations are generated provides a large reduction in the number of feasible folded forms. We have been developing schemes that limit the conformations generated simply by forcing them to be within a small volume (Jernigan and Covell). It has been possible to enumerate all of the possible folded topologies of several small proteins and evaluate them with simple residue-residue interactions. Success with this evaluation reduces the number of important conformers by a further factor of ten. Folded proteins and RNA bear a resemblance in that both are densely packed structures. In this way it should be feasible to consider the folded conformations of both small proteins and RNA of unknown structure.

Molecular modeling has been proceeding in four areas: membrane channels (Guy and Raghunathan), small peptides (Guy, Jernigan, and Covell). DNA double helix (Jernigan, Sarai, Mazur, Nussinov, Shapiro, and Owens), and DNA-protein interactions (Takeda and Sarai). 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. Conformational models have been developed for the antibiotic magainin, pardaxin, ô lysin, and PGLa; these have assisted in understanding how they can lyse cells and form channels. Small peptide 2D NOE NMR data indicating close atoms has been combined with molecular calculations to yield models for the neuropeptide Substance P. DNA double helices exhibit some dependence on the base sequence; these details are being studied by investigating the sequence dependence of the DNA helix flexibility. Methods to calculate the induction of bends of specific shapes and curvatures are being developed. One question that is unresolved is whether or not these conformations play a functional role in gene regulation. For the DNA-protein interactions in operator-repressor interactions, the modelling proceeds by evaluating the strengths of various classes of interactions. Comprehensive experiments in which repressor-operator binding is perturbed by changing the base sequence with every possible single base pair substitution and a number of amino acid substitutions (Takeda and Sarai) provide information about the strengths of the individual atomic DNA-protein interactions and how to best place the repressor in its most favorable orientation within the major groove of the operator region. Molecular computer graphics systems enhance all of these modeling efforts (Ting).

In a simple attempt to understand DNA conformations in control regions, one of the simplest aspects of this process, namely, the strength of strand pairing in the promoter region (Margalit, Shapiro, Nussinov, Owens, and Jernigan) has been studied. Stability of the DNA double helix was determined in the vicinity of

the promoter by computing the free energy for strand separation with dinucleotide free energy values taken from reported calorimetric measurements. For a large set of prokaryotic promoters we find the region just before mRNA start sites to be significantly less stable. A comparison of the free energies of promoter mutations indicates a strong correlation between the free energy change and the mutation type in this region.

Image Analysis. There are three areas of research interest in the Image Processing Section (Lipkin): 1) development of analytic methods to analyze two dimensional gels (Lemkin), 2) nucleic acid secondary structure studies (Shapiro) and 3) the hardware and software support (Schultz). Extensions to the program GELLAB have been utilized in the verification of two secreted axonal proteins in a neural culture system (Lemkin and Sonderegger). There is also a study of growth proteins that have been identified as growth or differentiation markers. Conversion of essential programs, as needed for newer computers, has also progressed. There has been substantial progress in developing an integrated computer environment to enhance RNA secondary structure programs (Shapiro). This system is expected to form the basis for an "expert system" to handle queries about RNA secondary structures. Algorithms are being developed to speed the predictions of secondary structures and to compare predicted structures. This latter method utilizes general tree matching considerations.

Theoretical Immunology. The programs of the Theoretical Immunology Section (Weinstein) focus on monoclonal antibodies. The aims are: 1) to establish a framework of pharmacological information with which to guide the use of monoclonal antibodies in vivo, 2) to exploit that framework in the development and use of cytotoxic antibody conjugates, 3) to exploit the framework of information in the design, conduct, and analysis of clinical trials with monoclonal antibodies, and 4) to develop criteria for the rational design of new-generation "immunoreactive agents" that will then be produced by recombinant techniques. The work of the Section is approximately equally theoretical and experimental.

The feasibility of delivering monoclonal antibodies via lymphatic vessels for diagnosis was demonstrated ("immunolymphoscintigraphy") and possible treatment ("immunolymphotherapy") of lymph node metastases and lymphoma (Black, Talley, and Eger). The initial studies were done in animals, using antigenic targets on both normal and malignant cells. Using the SAAM program package, kinetic models (Covell and Eger) were then developed. The Section has been collaborating with other groups at NIH on trials (10 total) of lymphatic delivery in melanoma, T-cell lymphoma, small cell carcinoma, non-small cell carcinoma, and breast cancer. The initial goal is a sensitive, non-surgical diagnostic method. The clinical study on T-cell lymphoma has produced the most efficient imaging of tumor cells yet achieved in humans by any non-invasive technique.

Four related programs in the Theoretical Immunology Section are: 1) The "percolation" problem: For tumor cells far from the nearest lymphatic or blood vessel, binding of antibody 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 the partial differential equations. The analyses take 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. 2) Immunotoxins and alpha-emitter/antibody conjugates (Black): These have been developed and then applied both in vitro and in vivo. Studies in mice have produced the first selective ablation of target cells yet achieved in vivo with an alpha-emitter conjugate. 3) Structural concomitants of antigenicity: Three algorithms (HAL, HALP, and HALCO) have been developed with which to analyze secondary structural characteristics of peptides and to assess their potential for antigenicity or for other functional characteristics. The results have been applied to HTLVIII/LAV envelope protein. 4) Liposomes and monoclonal antibodies are being used for targeted delivery of anti-viral agents to HIV-infected cells.

Membrane Structure and Function. The research goals in the Membrane Structure and Function Section (Blumenthal, Morris, Grimaldi, Eidelman, Puri, Sarkar, de Graca Miguel, and Paternostre) are directed towards an understanding of mechanisms of membrane fusion mediated by viral spike glycoproteins. The mode of action of the G protein of Vesicular Stomatitis Virus (VSV), the HN and F proteins of Sendai Virus, and the HA protein of influenza virus are studied. Specific topics include: 1) development of fluorescent methods to study kinetics and extent of adhesion and fusion using intact and reconstituted virions, and liposomes and cells as targets; 2) development of methods to analyze reconstitution of viral spike glycoproteins; 3) functional reconstitution of viral spike glycoproteins into lipid vesicles; 4) studies of mechanism based on an allosteric model: the role of ligand binding, conformational changes and cooperativity of viral spike glycoproteins in mediating membrane fusion; 5) studies of the effects of modifications of viral spike glycoproteins by pH temperature, enzymes, and chemicals on their fusogenic activities; 6) studies of the relationship between virus-induced membrane destabilization (permeability changes) and fusion; 7) Studies of viral entry into the cell by endocytosis using fluorescent techniques; 8) Application of image processing using video-enhanced fluorescent microscopy controlled by a computor to analyze viral entry pathways; 9) Examination of the disposition of the fusion protein after the fusion event; 10) Identification of possible fusion intermediates; 11) Development of methods to study fusion activity of mutants of viral proteins using cloned viral membrane protein sequences expressed in transfected cells; 12) Structural studies of viral proteins; 13) Development of methods for using reconstituted viral envelopes as vehicles for specific delivery of materials into cells.

Membrane Biology. The activity of the Membrane Biology Section (Pinto da Silva, Andersson-Forsman, Fujimoto, and Pimenta) is focused on the discovery, development and application of a new method for the visualization of cell and membrane surfaces at macromolecular resolution. This "Fracture-Flip" method is based on the splitting of biomembranes by freeze fracture and on the stabilization of the exoplasmic halves of split membranes by carbon evaporation. Briefly, after freeze-fracture, the specimen are not replicated by platinum evaporation but, instead, are stabilized by the condensation of a thin, electron-translucent film of carbon. After thawing and washing away unfractured cells in suspension, the carbon casts, together with their attached freeze-fractured membranes, are picked up on formvar coated grids and inverted, i.e. "flipped". A high resolution electron dense cast of the actual membrane surfaces, now on top, is produced by condensation of Platinum evaporated from an electron gun. The results obtained confirm the truly exceptional promise of

Fracture-Flip. Routinely, the resolution is one to two orders of magnitude better than that of high resolution scanning electron microscopy. When combined with pre- or post-fracture immunogold-labelling cytochemistry, fracture-flip allows the localization of specific antigens and/or receptors to macromolecular structures on the cell surface. The exceptional promise of the new method led to concentration on the development and application of fracture flip and of fracture-flip cytochemistry. Studies completed include: the ultrastructure of the surfaces of blood cells, the dynamics of endocytosis during the adhesion of macrophages to glass, the ultrastructure of the surface of spermatozoa, the visualization of inner surfaces of membranes, ultrastructure of the inner surface of the zonula occludens, ultrastructure of the cytoplasmic and exoplasmic surfaces of the nuclear envelope (including the nuclear pore complex), surface ultrastructure of nerve cells in culture, and comparative ultrastructure of infective and non-infective stages of Leishmania parasites. There are current investigations of membrane dynamics during release of mast cell granules, the localization of the GABA receptor in nerve cells in culture, the macromolecular ultrastructure of human lymphocytes during and after capping of HL-A antigens (together with Pavan, Univ. Rome), and the localization of the infection-specific glycolipid in Leishmania parasites. Other projects have included development of post-replication label-fracture, the application of label-fracture cytochemistry to study cell capping, and conventional freeze-fracture to examine side views of the zonula occludens.

Simulation, Analysis and Modelling of Physiological Systems. Development continued on the simulation, analysis, and modeling (SAAM/CONSAM) computer programs (Zech and Boston). Both batch (SAAM) and interactive (CONSAM) versions were ported to the UNIX-PC workstation, making the capabilities of this software available on a computer costing less than $5,000. A new graphics capability was added to the CONSAM programs in the form of a virtual terminal written in the C language through the use of a graphic kernal standard (GKS) binding. Development also continued on the theoretical development of parameter identifiability and estimability. The SAAM project participated in the an international committee to contribute advice regarding the direction of kinetic analysis by serving as the chairman of the advisory committee for a resource in kinetic analysis. The SAAM group organized and participated in workshops on the topics of SAAM/CONSAM in kinetic analysis. The "SAAM NEWS" was written and distributed to more than 650 interested members of the international user community. More than 100 copies of SAAM/CONSAM were distributed, with manuals and user guides, in an effort to foster the collaborative efforts deemed necessary by the scientific counselors. The SAAM project also carried out collaborative research efforts, involving a large number of national and international investigators, in the analysis of data in the fields of IgE metabolism, omega-3 fatty acid modulation of triglyceride metabolism, the quantitative description of chylomicron triglyceride and cholesterol ester metabolism, the contribution of plasma lipoprotein cholesterol, and cholesterol ester metabolism to the formation of hepatic cholesterol and bile acid metabolism, the testing of hypothesis testing of concerning human epinepherine and norepinepherine metabolism, and the quantitative description of the whole body metabolism of cancer perventive selenium compounds.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08300-16 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

SAAM, Development and Applications for Analogic Systems Realization

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

Loren A. Zech, M.D.,	Senior Investigator Detail from OD, NIHLB	LTB, NCI

Other Professional Personnel:

Kevin C. Lewis, Ph.D.	IRTA Staff Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Ray Boston, Murdoch Univ.,Australia; Dr. Charles Schwartz, Medical College of Virginia, Richmond, VA; Dr. Waldo R. Fisher, Univ. of Fla., Gainesville; Drs. H. Bryan Brewer, Richard E. Gregg, Carlo Gabelli, Dubo Bojanovski, Juergan Schaefer,

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.75	.75	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.)*

Continuing development of a computer system (SAAM) for the simulation, analysis, and modeling of bio-kinetic systems. In 1987-88 yr we advanced the SAAM/CONSAM extensions to allow for increases in the number of compartments, components, and adjustable parameters having previously accomplished the increase in the number of data points which can be used with the simulator. A "Unix (BSD 4.3)" version of SAAM29/CONSAM was implemented because of the number of users which have chosen to use this operating system and because of the anticipation of the conversion of the Laboratory of Mathematical Biology to this standard operating system. A 75 compartment version of SAAM and CONSAM has been developed using these new tools and is presently being tested for errors. A version of CONSAM29 making use of a virtual terminal was developed for the VMS versions of the program. The 30+, C-language, subroutines which makeup the virtual terminal utilize the Plot-10 GKS Fortran bindings. This implementation necessitated the addition of a graph name characteristic to the CONSAM plot command. CONSAM and the virtual terminal are executed as separate independent processes in which information and control can be passed from CONSAM to the virtual terminal on the plot command.

A compartmental model was developed to analyze the kinetics of distribution and metabolism of Norepinephrine. This model was used to accept the concept that the increase in plasma Norepinephrine concentration during sodium restriction in humans is not due to increases in sympathetic nervous activation as observed in rising from a supine position, but due to a decrease in the volume of distribution. This change in volume of distribution resulted in an apparent decrease in clearance rate as measured by the calculation of Metabolic clearence rate.

Insulin deficiency in alloxan induced diabetes of the rabbit resulted in a decrease in Low Density Lipoprotein fractional catabolic rate and an increase in residence time. Plasma LDL Cholesterol decreases in diabetic rabbits.

GPO 914-918

Cooperating Units (Continued):

Molecular Diseases Branch, NIHLB; Dr. Ernest Schaefer, USDA Human Nutrition Center, Tufts Univ, Boston, MA; Drs. Y-Da Chen, & Gerald Reaven, Stanford Univ., Stanford, CA; Dr. Phil Taylor, Cancer Prevention Studies Branch, NCI; Dr. Orville Lavender, Beltsville Human Nutrition Research Center, USDA; Dr. Blossom Patterson, Operations Research Branch, NCI; Dr. Barbara Howard, Medlantic Research Foundation, Washington D.C.; Drs. Oscar Lenaris Jeffery Halter, U of Michigan; Dr. Ba-Bie Teng & Allen Sniderman, Royal Victoria Hospital, Montreal, Quebec; Dr. Gilbert Thompson, Hammersmith Hospital, London, England; Dr. David M. Foster, University of Washington, Seattle, WA.

Project Description #1:

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.

Major Findings:

(1) Computational Procedures: Continuing development of a computer system (SAAM) for the simulation, analysis, and modeling of bio-kinetic systems. In 1987-1988 yr. we advanced the SAAM/CONSAM extensions to allow for increases in the number of compartments, components and adjustable parameters having previously accomplished the increase in the number of data points which can be used with the simulator as well as an extension to 30 compartments.

A "Unix (BSD 4.2)" version of SAAM29/CONSAM was implemented because of the number of users which have chosen to use this operating system and because of the anticipation of the conversion of the Laboratory of Mathematical Biology to this standard operating system. Testing of a version of SAAM29/CONSAM29 which was developed for the UNIX-PC was completed.

Because the UNIX-PC functions as both the computer and terminal, software for a virtual terminal was added to CONSAM. The 30+, C-language, subroutines which makeup the virtual terminal utilize the GSS-GKS C bindings. This implementation necessitated the addition of a graph name characteristic to the CONSAM plot command. CONSAM and the virtual terminal are execute as separate independent processes in which information and control can be passed from CONSAM to the virtual terminal on the plot command. Using PLOT-10 GKS (a GKS implementation from Tektronics with a Fortran binding) the virtual terminal graphics utility was transferred to the VAX series of computers. This graphics is implemented in a new version of CONSAM designated CONSAM29G. This version of CONSAM contains drivers for all Tek 4100 and 4200 series terminals.

The parameterization of the SAAM and CONSAM source code has continued and the size of the SAAM and CONSAM objects can now be specified by a small 'base set' of parameters at computation time. This 'base set' of parameters contains the the number of compartments and the number of data points and several other parameters which can not be derived from these two. This parameterization has

been tested by generating a 30 compartment/500 data point excitable image, a 50 compartment/750 data point excitable image, and a 75 compartment/100 data point excitable image. A each of these milestones it was necessary to make substantial improvements to the data structures associated with the source code. A 75 compartment/1000 data point version of SAAM/CONSAM designated SAAM30 has been been distributed to a handful of beta-test users through the Resource For Kinetic Analysis.

A public domain spread-sheet SC was added to CONSAM as a subcommand (much as the insert mode of the editor functions as a subcommand). The spread-sheet cells are softly linked by storing the name of a SAAM parameter such that values can be selectively retrieved at the users discretion. CONSAM commands can be issued from within the spread-sheet subcommand. A new command was added to CONSAM, spread-sheet, which calls the spread-sheet subsystem. On exit from the spread-sheet, control returns to CONSAM. This spread-sheet was implemented in the UNIX BSD 4.3 version of CONSAM for testing before implementation on the VAX/VMS system.

When data is avaliable from several related studies, it is frequently desired to use these multiple studies to make population estimates of model parameters. This combination of results not only gives a better statistical result because of increased sample size, but provides the statistical power to detect possible differences between populations (i.e. treated vs control). SAAM has for some time had the ability to perform multiple study analysis, through the invocation of 'Model code 11', an implementation of the Pettigrew methodology. A facility has been added to SAAM to aggregate the results of multiple studies such that the results could be recalled as a group and processed using 'Model code 11'.

(2) SAAM Workshops, Distribution, & Newsletter: One workshop was conducted for 10 new users of SAAM and CONSAM at the FCRF on 27 and 28th of June. This workshop was composed of 8 hours of lectures and 8 hours of practice on the computer. A four hour symposium was conducted at FASEB consisting of four one hour lectures devoted to the use of SAAM and CONSAM in the analysis of Nutritional problems.

A two day international SAAM USERS meeting (September 21 and 22) with workshops, tutorials, and presentations of work in progress will be conducted in conjunction with the Resource for Kinetic Analysis. Approximately 100 participants are expected to attend. This represents the forth users meeting, previous user meetings being conducted in 1987 1976 and 1982. SAAM29G and other new versions of SAAM/CONSAM will be announced at this meeting.

Over 200 copies of the SAAM/CONSAM software have been given to the scientific community over the past 12 months in an effort to establish other centers in the collaborative effort, as pointed out by the scientific counselors as necessary. This involves combining and confronting theorist and experimentalist with topics which can profit from the application of computer simulation and computation and further serves to obtain the best experimental data for analysis an inclusion in data bases, such as the lipoprotein and

selenium data bases. To this end we plan to continue to distribute these programs as necessary in the scientific community.

The forth, fifth and sixth SAAMNEWS letters were written with the Resource for Kinetic Analysis which sent each newsletter to over 600 investigators who had indicated that they had an interest in receiving such a news letter.

Project Description #2:

Project #2 Application of SAAM and CONSAM to the Simulation and Analysis of Bio-kinetic Data.

Major Findings:

(1) In collaboration with Dr. Fisher and Dr. Stacpoole a detailed investigation and analysis of the affects of omega-6 (safflower oil) and omega-3 (fish oil) fatty acid-enriched diets (65% carbohydrate, 20% fat) in two patients with a syndrome of diabetes mellitus, lipodystrophy, acanthosis nigricans, chylomicronemia and abdominal pain. 3H-glycerol was used to evaluate very low-density lipoprotein-triglyceride (VLDL-TG) metabolism, and changes in glucose and insulin dynamics were also studied. On the omega-6 diet, both subjects demonstrated four to five times normal rates of VLDL-TG production and glycerol biosynthesis, and striking decrements in the fractional catabolic rate (FCR) for VLDL-TG and VLDL-particles. Both subjects had elevations in non-esterified fatty acid (NEFA) concentrations. In one patient, the omega-3 diet markedly decreased plasma triglycerides and newly synthesized triglyceride glycerol production, in association with a fall in NEFA. In both subjects, plasma glycerol reutilization for triglyceride synthesis, normal on the omega-6 diet, was abolished on the omega-3 regimen. Plasma postheparin lipolytic activity was normal on both diets. On the omega-3 diet, xanthomas and hepatomegaly decreased and, in the patient who had no reduction in serum triglycerides, pancreatitis attacks virtually ceased. Mean 24h serum glucose levels were higher and both basal and peak C-peptide responses to a carbohydrate meal were blunted on the omega-3 diet. One patient became ketonuric. We conclude the major cause of hypertriglyceridemia in these patients was due to increased lipid synthesis and hypothesize that this is secondary to high plasma concentrations of NEFA. In addition, an omega-3 diet in these subjects inhibited insulin secretion and worsened glucose tolerance.

(2) In collaboration with Dr. Schwartz, in vivo transport of free cholesterol and esterified cholesterol among the components of blood was investigated. Five experiments were carried out in normo-lipidemic subjects administered strategic combinations of two of the following; ^{14}C mevalonic acid, LDL free ^{3}H or ^{14}C cholesterol, VLDL free ^{3}H and HDL free ^{3}H or ^{14}C cholesterol; frequent blood samples were then obtained for up to two days. The mass and specific activity of HDL-FC, HDL-EC, β-(LDL/VLDL)-FC, β-(LDL/VLDL)-EC, and erythrocyte FC were used to construct a compartmental model using the SAAM program. The kinetic analysis showed that LDL-FC and VLDL-FC were transported in an identical fashion. Erythrocyte FC conformed to a single pool which exchanged in a bidirectional fashion with both HDL and LDL/VLDL without evidence of net

FC transport. The plasma concentration of HDL-FC was less than 30% of LDL/VLDL-FC but erythrocyte FC exchange with HDL substantially exceeded that with LDL/VLDL (10 vs 4.4 μmol/min respectively).

The LDL/VLDL EC data was resolved in each subject by transport of EC from HDL to LDL/VLDL (range, 7-18 umol/min). There was considerable transport of EC from LDL/VLDL back to HDL but the net transport of EC was from HDL to LDL/VLDL. The range of net transport was 2.9-3.4 μmol per min which equaled the loss of EC from plasma via LDL/VLDL; HDL-EC was not directly removed from plasma. The fact that HDL-EC was not transported directly out of plasma to tissue is consistent with the concept that HDL is not degraded as an intact particle. The finding that HDL mediates extensive FC exchange between plasma and tissue without transfer of EC to tissue indicates a unique mechanism of HDL-plasma membrane interaction.

(3) In collaboration with Dr. Linares and Dr. Halter; we used compartmental analysis to analyze plasma norepinephrine (NE) kinetics to determine whether the increase in plasma norepinephrine (NE) concentrations during sodium restriction in humans is due to sympathetic nervous system (SNS) activation with increased NE release or due to a decrease in its metabolic clearance rate (MCR1). ^{3}H-norepinephrine was administered (15 μCi/m^2 bolus + 0.35 uCi/min/m^2 infusion for 50 min. and 20 min. postinfusion decay) to a group of healthy young subjects in the supine position (N=10) and during 60 min. of standing (N=7), during normal-sodium diet and after 7 days of 10 mEq/day sodium-restricted diet. The mean supine plasma NE concentration was greater during sodium-restricted diet compared to normal-sodium diet (154±9 vs 185±12 pg/ml, p=0.02, N=10). During both normal-sodium and sodium-restricted diets, upright plasma NE concentrations increased (163±4 vs 359±38 pg/ml and 182±8 vs 401±26 pg/ml, respectively, MANOVA p<0.001, alpha=0.05).

The increases of plasma NE concentration with both sodium-restricted diet and upright posture were accompanied by a fall in MCR1 associated with a fall in the volume of distribution of NE (6.1±.4 vs 5.0±.4 liters, p=0.003, N=10). However, in contrast to the effect of upright posture to increase NE release into the extravascular compartment, during sodium-restricted diet there were no changes in NE release rate (1.63±.09 vs 1.62±.1 μg/min/m^2, p=0.97, N=10), or the mass of NE in either compartment 1 (0.48;.03 vs 0.50±.05 ug/m^2, p=0.56, N=10) or compartment 2 (81±18 vs 88±24 μg/m2, p=0.46, N=10). We conclude that the increase in supine plasma NE concentration during sodium-restricted diet in humans is due to a fall in the volume of distribution of NE resulting in a decrease in its metabolic clearance rate, not to an increase in sympathetic nervous system activation. Compartmental analysis of NE kinetics provides information, not easily obtainable by other methods, about NE metabolism in the study of human noradrenergic system physiology.

(4) In collaboration with Dr. Y-D Chen, and Dr. Gerald Reaven; studies have been carried out in rabbits with allaxon-induced diabetes in order to see if insulin deficiency affects LDL catabolism. The results indicated that plasma LDL-cholesterol was lower in diabetic rabbits, associated with a fall in the cholesterol to protein ratio of LDL lipoprotein particles. In addition, ^{125}I-LDL disappeared more slowly from plasma of diabetic rabbits, leading to a

significant reduction in fractional catabolic rate and a decrease in residence time of ^{125}I-LDL. These data demonstrate that LDL composition and catabolism are altered as a consequence of insulin deficiency.

(5) In collaboration with Drs. Ba-Bie Ting, Gilbert Thompson, Peter Kwiterovich, and Allen Sniderman LDL ApoB turnover kinetics has been re-examined. LDL ApoB kinetics have generally been analyzed by the two compartmental model of mathues, the assumption being that all plasma LDL particles have an equal probability of being catabolized. LDL has been shown to be both structurally and kinetically heterogenous in a wide verity of studies leading to a major contradiction of the excepted model. Accordingly a new multicompartmental model of LDL metabolism was developed and tested using data from one normal and three hyperapoB subjects. Each received a simultaneous injection of ^{125}I-buoyant (L-LDL) and LDL^{131}I-dense LDL (H-LDL). A model was developed containing, 1)a L-LDL delipidation cascade with a slowly catabolized pool which is derived from the cascade; 2) pathways where the L-LDL is converted to H-HDL but a recirculation pathway where H-LDL can return to the liver and subsequently re-enter the plasma space as L-LDL; and 3) L-LDL and H-LDL can each be directly catabolized. Quantitative analysis using this model resulted in the calculation of a fractional catabolic rate for L-LDL which was one third the fractional catabolic rate for H-LDL apoB (1.33 vs .42, /day). On the average 62% of the L-LDL apoB moves through the cascade to act as precursor for H-LDL (36% to 90%). The model underscores the physiologic importance of cholesterol-ester exchanges in the production and metabolism of H-LDL and L-HDL. Several additional normal and familial hypercholesterolemic subjects have been examined in the past year to test the proposed model, resulting in conformation of the new LDL model.

(6) In collaboration with Dr. Blossom Patterson, Operations Research Branch, NCI; Dr. Phil Taylor, Cancer Prevention Studies Branch, NCI; Dr. Orville Lavender, Beltsville Human Nutrition Research Center, USDA; we are investigating selenium kinetics. Little is currently known about the kinetics of selenium in humans. The Selenium Pharmacokinetics Study a joint study between NCI and USDA was designed to estimate basic pharmacokinetic parameters for two prototype forms of selenium: sodium selenite (inorganic) and selenomethionine (organic). Further, there was interest in whether these parameters would vary if either form was administered in a fasting or a non-fasting state as selenium is thought to be a cancer preventative in the proper dose and form.

The metabolism of selenium appears much more complex than was originally thought when the study was designed. This has led to kinetic modelling. The main study was preceded by a pilot study. Six subjects were each given a single, oral dose of 200 micrograms of sodium selenite. The cold stable isotope, ^{74}Se, was used as a label for the dose. These six sets of data are being used to realize a model for selenium metabolism.

In the main study thirty-two subjects were each given a single oral labeled dose of selenite or selenomethionine. Subjects were on a controlled diet for three days prior to dosing, and twelve days thereafter. This allowed us to determine their total intake of selenium, and helped assure that they would be

in steady state. A split unit design was chosen for the study. Subjects were randomized in equal numbers to receive either selenite or selenomethionine. Each person received a single substance, in both nutritional states. This design was chosen to allow precise measurement of any differences resulting from fasting state, while minimizing the number of subjects required. Sample size methodology for experimental designs in which more than two groups are being compared was adapted to a split unit design. This required making some simplifying assumptions. This methodology is being pursued as it has applicability to future studies. This study has a power of about 75% to detect differences in selenium levels of one standard deviation. The data consist of measures of labeled selenium in the plasma, the red blood cells, the urine and the feces. At present, laboratory analysis of samples is close to complete.

Data analysis has centered around estimation of those parameters necessary to make decisions about size and frequency of dosing. Our analysis up to now has been based primarily on pilot study data. We began our analysis by investigating the hypothesis that a simple model - a single compartment model - would fit the data. We quickly realized that this was not the case.

At the present time we have testing a selenium model using the pilot study data which has four dissimilar plasma components, representing possibly three or four different selenium compounds. The plasma data for all six pilot study subjects show similar findings. In addition, the plasma and urine data could not be explained together by a simpler model. The goal of kinetic modeling is to develop a compartmental model consistent with all the data, considered simultaneously. Such a model allows the making of inferences about the metabolism of a substance, and its distribution in the body pools. Presently the model has first, distributed absorption from several compartments in the small intestine. Secondly, there is enterohepatic recirculation. Thirdly, selenoproteins are secreted by the splanchnic bed (probably the liver secreting glutathione peroxidase) back into the plasma which are finally taken up in the tissue pools which exchange with the plasma and secrete metabolic products.

Our current model is complex; it has four plasma components, and it has 25 compartments when all the tissues are represented; however, it appears to be the simplest model to fit the pilot study data quite. We're in the process of making minor modifications to it and then plan to use it as a hypothesis to examine the main study data.

Over the past year we have applied this model to fasting and nonfasting main study subjects receiving inorganic selenium compounds. There appears to be a difference in the two most rapid plasma components. A statistically significant but nutritionally unimportant difference in absorption between the fasting , 80.2±, and non-fasting, 76∫, subjects was detected. In the feed state a larger fraction of the absorbed tracer dose is removed by the liver before appearance in the plasma. This response may be secondary to increased portal flow associated with eating. If specific plasma components prove to be important in cancer prevention, this pharmacokinetic analysis will provide optimal conditions for dosing. A differentiation between the dilivery of chemo-preventive selenium to the liver and peripheral tissues is modulated by the fasting status of the study subject.

A pilot model for the analysis of the kinetics organic selenium, selenomethionine has been developed using the first four studies. This model will be used to compare the kinetics of organic selenium compounds and its modulation by fasting and nonfasting status of the study subjects.

These models will allow us to make population estimates of the various pharmacokinetic parameters. Finally, these estimates will be compared using analysis of variance techniques. Both main effects and interactions of form selenium and fasting state will be investigated.

Publications:

Gregg RE, Zech LA, Schaefer EJ, Stark D, Wilson D, Brewer HB, Jr. Abnormal in vivo metabolism of apolipoprotein E_4 in humans. J Clin Invest 1986;78:815-821.

Bojanovski D, Gregg TE, Zech LA, Meng MS, Bishop C, Ronan R, Brewer HB, Jr. In vivo metabolism of proapolipoprotein A-I in Tangier Disease. J Clin Invest. 1987;80:1742-1747.

Brewer HB, Gregg RE, Law SW, Hoeg JM, Zech LA. Human apolipoprotein A-I and A-II metabolism. In: Paoletti R. et. al. (ed). Drugs affecting lipid metabolism. Berlin: Springer Verlag, 1987;236-243.

Linares OA, Jacquez JA, Zech LA, Sanfield JA, Marrow LA, Rosen SG, Halter JB. Norepinephrine metabolism in humans: A kinetic analysis and physiologic model. J Clin Invest. 1987;80:1332-1341.

Gregg RE, Zech LA, Gabelli C, Hoeg JM, Brewer HB, Jr. The role of Apolipoprotein E and the low-density lipoprotein receptor in modulating the in vivo metabolism of apolipoprotein-B-containing lipoproteins. In: Gallo LL, ed. Cardiovascular Disease. NY: Plenum Publishing Co. 1987; Chapter 11, 93-102.

Zech LA. Metabolic channeling of ApoB containing lipoproteins: Or analogic realization of the ApoB dynamical system. In: Lippel K, ed. Proceedings of the workshop on lipoprotein heterogeneity. United States Government Printing Office, NIH Publication No. 87-2646;237-247.

Linares OA, Zech LA, Jacquez JA, Rosen SG, Sanfield JA, Marrow LA, Supiano MA, Halter J.B. Effect of sodium-restricted diet and posture on norepinephrine kinetics in humans: A compartmental analysis. Am J Physiol. 1988;254:E222-E230.

Golay A, Zech LA, Shi MZ, Jeng CY, Chiou Y-AM, Reaven GM, Chen Y-D. Effect of insulin defficiency on low density lipoprotein metabolism in rabbits. Hormone and Metabolic Res. 1988;20:11-14.

Foster DM, Boston RC, Jacquez JA, Zech LA. Resource facility in kinetic analysis: Modeling using the SAAM computer programs. Health Physics 1988. In Press.

Foster DM, Boston RC, Zech LA. Kinetics of lipoprotein metabolism: Special considerations in modeling. IFAC Proceedings, NY: Pergamon Press 1988. In Press.

Stacpoole PW, Alig J, Kilgore LL, Ayala CM, Herbert PN, Zech LA, Fisher WR. Lipodystrophic diabetes mellitus. Investigation of lipoprotein metabolism and the effects of Omega-3 fatty acid administration in two patients. J Clin Endo and Metab. In Press.

Gregg RE, Zech LA, Gabelli C, Brewer HB, Jr. Apolipoprotein E regulates the catabolism of apolipoprotein B in humans. Steinmetz A, Kaffarnik H. eds. In: Proceedings of the Second Marburg Conference on Lipoproteins. In Press.

Redda K, Walker C, Barnett G. eds. Cocaine, Marijuana and Designer Drugs: Chemical, Pharmacological and Behavioral Aspects. Boca Raton: CRC Press, Inc. In Press.

Barnett G, Redda K, Kapaka R. Designer drugs: An overview. In: Redda K, Walker C, Barnett G. eds. Cocaine, Marijuana, and Designer Drugs: Chemical, Pharmacological and Behavioral Aspects. Boca Raton: CRC Press, Inc. In Press

Chiang CN, Barnett G. Marijuana: Pharmacokinetics and pharmacodynamics. In: Redda K, Walker C, Barnett G. eds. Cocaine, Marijuana, and Designer Drugs: Chemical, Pharmacological and Behavioral Aspects. Boca Raton: CRC Press, Inc. In Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08303-16 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Membrane Fusion Mediated by Viral Spike Glycoproteins.

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:

Stephen J. Morris Ph.D.	Expert	LTB, NCI
Ofer Eidelman, Ph.D.	Visiting Associate	LTB, NCI
Anu Puri, Ph.D.	Visiting Fellow	LTB, NCI
Debi Sarkar, Ph.D.	Visiting Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Joel Lowy, NHBLI; Dr. Michel Ollivon, CNRS, France; Dr. Abraham Loyter, Hebrew University, Israel; Dr. Joshua Zimmerberg, NIDDK

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Structure & Function Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The research goals in the Membrane Structure and Function Section are directed towards an understanding of mechanisms of membrane fusion mediated by viral spike glycoproteins. We are specifically studying the mode of action of the G protein of Vesicular Stomatitis Virus (VSV), the HN and F proteins of Sendai Virus, and the HA protein of influenza virus. Specific topics include: i) development of fluorescent methods to study kinetics and extent of adhesion and fusion using intact and reconstituted virions, and liposomes and cells as targets; ii) development of methods to analyze reconstitution of viral spike glycoproteins; iii) functional reconstitution of viral spike glycoproteins into lipid vesicles iv) studies of mechanism based on an allosteric model: the role of ligand binding, conformational changes and cooperativity of viral spike glycoproteins in mediating membrane fusion v.) studies of the effects of modifications of viral spike glycoproteins by pH temperature, enzymes, and chemicals on their fusogenic activities vi) studies of the relationship between virus-induced membrane destabilization (permeability changes) and fusion vii.) Studies of viral entry into the cell by endocytosis using fluorescent techniques. viii.) Application of image processing using video-enhanced fluorescent microscopy controlled by a computer to analyze viral entry pathways. ix) Examination of the disposition of the fusion protein after the fusion event; x) Identification of possible fusion intermediates; xi) Development of methods to study fusion activity of mutants of viral proteins using cloned viral membrane protein sequences expressed in transfected cells; xii) Structural studies of viral proteins; xiii) Development of methods for using reconstituted viral envelopes as vehicles for specific delivery of materials into cells.

GPO 914-918

Project Description:

Other Professional Personnel (continued):

M.T. Paternostre, Ph.D.	Guest Researcher	LTB, NCI
Maria da Gracia Miguel, Ph.D.	Visiting Fellow	LTB, NCI
Settimio Grimaldi Ph.D.	Visiting Scientist	LTB, NCI

Major Findings:

1. We developed a new spectrofluorometric assay for measuring fusion between membranes of 3T3 fibroblasts expressing influenza virus hemagglutinin (HA) and human red blood cells (RBC) with a 1 second time resolution.

2. We found that fusion was a rapid and highly cooperative process. The maximal extent was reached within minutes, there was a measurable (about 30s) lag phase, and temperature-dependence suggests a two or more step process.

3. Fusion occured with dead cells indicating that neither metabolic energy, nor gradients of pH, membrane potential or osmotic pressure play a role in fusion of RBC to HA-expressing fibroblasts.

4. Fusion initiated by lowering the pH could not be arrested by bringing the pH back to neutral after 15 s. This is consistent with an irreversible conformational change observed with HA.

5. We have extended our allosteric model for viral protein-mediated fusion to account for the cell fusion data. The model involves "nucleation" of a number of activated viral spike glycoprotein oligomers to form a "fusion complex". Equations based on the model describe well the observed delay in HA-induced cell fusion kinetics.

6. We have successfully reconstituted the VSV-G protein in lipid vesicles by solubilizing with the detergent TritonX100 followed by removal of the detergent in the presence of SM2 biobeads. Fluorescence probes were incorporated into the virosomes during reconstitution. Fusion of the virosomes with Vero cells was monitored by spectrofluorometry and fluorescence microscopy. The pH profile of virosome-Vero cell fusion paralleled that observed for intact VSV—cell fusion.

7. To develop a basis for rational reconstitution protocols the temperature dependence of self-association of a detergent, octylgulcoside (OG), a lipid, phosphatidylcholine (PC), and the VSV-G protein were examined using fluorescence techniques. We found that temperature manipulation alone will not permit co-existence of monomeric VSV-G and micellar lipid with the detergent.

8. In order to study the role of specific phospholipids in fusion of Vesicular Stomatitis Virus (VSV), we examined the interaction of the virus with Human red blood cells (RBC) using a spectrofluorometric assay. VSV bound to RBC in which the phospholipid asymmetry had been abolished, showed a rapid pH-dependent

fusion, whereas little or no fusion was observed with RBC whose asymmetry was intact. We concluded that one or more components of the inner surface of the RBC membrane might serve as a "receptor" for fusion with VSV.

9. Treatment of VSV was with Neuraminidase leads to significant enhancement in fusion to Vero cells, without affecting virus binding. Whether the sialic acid on the G protein retards fusion due to its charge or whether it affects the pH-induced conformational change of the protein still needs to be determined.

Publications:

Blumenthal R. Membrane fusion. Curr Top Membr Transp 1987;29:203-254.

Blumenthal R, Bali-Puri A, Walter A, Covell D, Eidelman O. pH-Dependent fusion of vesicular stomatitis virus with vero cells: Measurement by dequenching of octadecylrhodamine fluorescence. J Biol Chem 1987;262:13614-13619.

Cafrey M, Morris SJ, Feigenson GW. Uranyl acetate induces gel phase formation in model lipid and biological membranes. Biophys J 1987;52:501-505.

Puri A, Winick J, Lowy RJ, Covell D, Eidelman O, Walter A, Blumenthal R. Activation of vesicular stomatitis virus fusion with cells by pretreatment at low pH. J Biol Chem 1988;263:4749-4753.

Ollivon M, Eidelman O, Blumenthal R, Walter A. Micelle-vesicle transitions of egg phosphatidylcholine and octylglucoside. Biochemistry 1988;27:1695-1703.

Blumenthal R. Cooperativity in viral fusion. Cell Biophys 1988;1:1-12.

Blumenthal R, Puri A, Walter A, Eidelman O. pH-Dependent fusion of vesicular stomatitis virus with cells: Studies of mechanism based on an allosteric model. In: Ohki S, Doyle D, Flanagan T, Hui SW, Mayhew E, eds. Molecular Mechanisms of Membrane Fusion. NY: Plenum Press, 1988;367-383.

Loyter A, Citovsky V, Blumenthal R. The use of fluorescence dequenching methods to follow viral membrane fusion events. Methods Biochem Anal 1988;33:128-164.

Eidelman O, Blumenthal R, Walter A. The composition of octylglucoside-phosphatidylcholine mixed-micelles. Biochemistry 1988;27:2839-2846.

Morris SJ, Bradley D, Gibson GC, Smith PD, Blumenthal R. Use of membrane-associated fluorescence probes to monitor fusion of vesicles: Rapid kinetics of aggregation and fusion using pyrene excimer/monomer fluoresecence. In: Loew L, ed.: Spectroscopic Membrane Probes. Boca Raton, FL: CRC Press, 1988; I: chapter 7, 161-191.

Walter A, Eidelman O, Ollivon M, Blumenthal R. Functional reconstitution of viral envelopes. In: Wilschut J, Hoekstra D, Eds. Cellular membrane fusion: Fundumental mechanisms and applications of membrane fusion techniques. NY: Marcel Dekker, Inc., 1988 in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08320-13 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Peptide Conformations

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:		
David Covell, Ph.D.	Expert	LTB, NCI
H. Robert Guy, Ph.D.	Sr. Staff Fellow	LTB, NCI
Kai-Li Ting, Ph.D.	Computer Programmer	LTB, NCI

COOPERATING UNITS *(if any)*

Dr. J. Ferretti, Laboratory of Chemistry, NIHLB

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues XX☐ (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. However, these maps do not coincide with calculated ones. We intend to investigate the origin of discrepancies in order to improve molecular calculations.

NMR investigations of Substance P, a neuropeptide, indicate no dominant conformations in water, but in methanol it is highly ordered with a large number of 2-Dimensional NMR NOE cross-peaks. With these data, molecular models consistent with the experimental data have been developed. Subsequently additional data obtained have assisted in indicating which of the alternative conformations are most probable.

Experimental studies of a 13 amino acid fragment of Ribonuclease A indicated it to be a stable helix at low temperatures. The existence of such a small relatively stable structure has been surprising. We have been looking in detail at possible molecular interactions to try to understand the remarkable stability of such a small peptide as a helix in water.

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

Project Description

Major Findings:

The aim is to study the conformations available to short peptides and, in a systematic way, develop better conformational prediction methods. Studies of homologous proteins can indicate permissible substitutions of amino acids. In some cases, single substitutions of amino acids cause significant changes in conformation beyond those expected from existing theories; whereas in other cases similar substitutions cause no major changes. 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 and selection of peptides for study by experiment and calculation. In the latter case, measurements of intramolecular distances by 2D NMR and fluorescence energy transfer measurements are underway. Results from these serve as constraints in calculations of possible molecular conformations.

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 for single amino acids are substantially smoother and also, there are clearer distinctions between the beta domains of the different residues. Unfortunately there are still not sufficient data to obtain useful maps for all possible pairs of amino acids.

We intend to utilize these results together with molecular models to obtain a better understanding of the features observed here, but not obtained with the usual molecular calculations. This should then lead to refinements that improve the calculation methods.

NMR 2 dimensional NOE studies of most small peptides in water indicate no cross peaks, and hence no dominant interactions or dominant conformations other than those expected from the chemical bonds. In two cases, the C peptide fragment of Ribonuclease A in water at low temperature and Substance P in methanol, there are indications of substantially fixed conformations for these small peptides.

Models for both molecules are being developed in a logical way by using these experimental data as constraints on energy calculations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08335-12 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 charecters 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. Chief, Theoretical Immunology Section, LTB, NCI

Other Professional Personnel:

Janos Szebeni, M.D. Visiting Associate LTB, NCI
Gurupadappa Betageri, Ph.D. Visiting Fellow LTB, NCI
Mary Jane Talley, B.S. Biologist LTB, NCI

COOPERATING UNITS *(if any)*

Drs. Sharon M. and Larry M. Wahl, NIDR; Drs. Mika Popovic and Suzanne Gartner, LTCB, DCE.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.9	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues xx☐ (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. Current studies are directed toward HIV-infected cells.

(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 ppselectively 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.

These strategies are being applied to problems in the therapy of cancer and AIDS. With respect to AIDS, we have formulated liposomes containing anti-viral drugs which do not ordinarily enter cells but which can be carried into monocyte/macrophages by the liposomes.

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

Project Description

Major Findings:

Our objeives are to investigate the use of liosomes 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; (5) develop methods for selective cytotoxic effect on cells using liposomes containing drugs and bearing immunoglobulin on their surfaces; (6) as part of No. 5, to target HIV-infected cells.

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. (h) Liposomes bearing antibody directed against the class I histocompatibility antigen $H\text{-}2K^k$ selectively kill class I-bearing cells. Work with such liposomes and with immunotoxins has led us to a new cell biological hypothesis, the "neutral bypass" (see also project no. Z01 CB 08367-03 LTB).

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.

Publications:

Weinstein JN, Leserman LD. Liposomes as drug carriers in cancer chemotherapy. I:Goldman ID ed. The International Encyclopedia of Pharmacology and Therapeutics, Vol. 118: Membrane Transport of Anti-neoplastic Agents New York: Pergamon 1986;169-194.

Weinstein JN. Liposomes in the diagnosis and treatment of Cancer, In: M. Ostro, ed. Liposomes, New York: Marcel Dekker, 1987;277-338.

Quill H, Carlson L, Fox BS, Weinstein JN, Schwartz RH. Optimization of antigen presentation to T cell hybridomas by purified Ia molecules in planar membranes: Ia molecule polymorphism determines the antigenic fine specificity of the response to cytochrome c peptides. J. Immunol. Methods, in press.

Barbet J, Black CDV, Holton O.D. III, Weinstein JN. Specific toxicity to activated T and B lymphocytes of a ricin A immunotoxin directed against the class I MHC antigen H-2 K. J. Antibody, Immunoconjugates and Radiopharmaceuticals 1988, in press.

Mitchell MS, Weinstein JN. Biotechnology products in cancer therapy. Cancer Research, 1988, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08341-10 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Studies of Lipid-Protein and Protein-Protein Interactions in HIV

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

John N. Weinstein, M.D., Ph.D.	Chief, Theoretical Immunology Section	LTB, NCI
Other Professional Personnel:		
H. Robert Guy, Ph.D.	Senior Staff Fellow	LTB, NCI
Kai-Li Ting, Ph.D.	Computer Programmer	LTB, NCI

COOPERATING UNITS *(if any)*

Dr. R. Schwartz, LI, NIAID; Dr. G. Shearer, IB, NCI. Drs. J. Segrest & Arantharamaiah, U. Alabama, Birmingham

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	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.)*

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.

Statistical and mechanical algorithms (HAL, HALP, HALCO) were devised for evaluating amphipathic helical structures and more general structure-function relationships in proteins and peptides. This is being used to define issues of structure and immunogenicity with respect to HLA antigens and to the envelope polyprotein of HIV.

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

Project Description:

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, dil. 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. (11) Development of the HAL (Helical Amphipathicity Locator) algorithm and compute program set for analysis of structure/function relationships in proteins. Related packages further developed during the last year include HALP, HALPSTAT, HALCOMP, COPHAL and HALCO. (12) Application of these programs to the problem of finding T-cell antigentic peptides and other structural features of the HIV envelope protein. (13) Predicted peptides have been synthesized and other structural have undergone physical chemical and immunological testing.

Publications:

Weinstein JN, Blumenthal R, Klausner RD. Carboxyfluorescein leakage assay for lipoprotein-liposome interaction. In: Segrest JP, Albers JJ eds. Methods in Enzymol. A, London: Academic Press, 1986;28(A):657-668.

Weinstein JN. Liposomes in the diagnosis and treatment of cancer. In: Ostro M, ed. Liposomes, New York: Marcel Dekker, 1987:277-338.

Quill H, Carlson L, Fox BS, Weinstein JN, Schwartz RH. Optimization of antigen presentation to T cell hybridomas by purified Ia molecules in planar membranes: Ia molecule polymorphism determines the antigenic fine specificity of the response to cytochrome-c peptides. J. Immunol. Methods, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08359-07 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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. Chief, Theoretical Immunology Section LTB, NCI

Other Professional Personnel:

Christopher D.V. Black, Ph.D. Visiting Associate LTB, NCI
Mary J. Talley, B.S. Biologist LTB, NCI

COOPERATING UNITS *(if any)*

Dr. A. Keenan, Dr. S.M. Larson, LHM, CC; Dr. R. Parker, Dr. S. Sieber, DCCP; Dr. L. Liotta, Dr. G. Bryant, LP, DCBD; Dr. M. Lotze, Dr. R. Rosenberg, SB, DCT; Dr. J. Mulshine, NCI-NMOB, DCT.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.3	0.4

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 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. The approach was extended to include endoscopic techniques for reaching lymph node groups not accessible by subcutaneous injection. Imaging studies were followed up with attempts at therapy. For diagnosis of early metastatic tumor in the nodes, the lymphatic route can 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 has been applied to detection of lymph node metastases in clinical stage II malignant melanoma and cutaneous T-cell lymphoma (CTCL) (with very good results). Similar protocols have been approved for breast carcinoma, small cell lung carcinoma, and non-small cell lung carcinoma. Our studies of CTCL have produced the most efficient antigen-specific imaging yet achieved in humans by any techniques.

In vitro and animal studies are being continued to optimize the clinical procedures (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.

Project Description:

Major Findings:

(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.

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 in patients with cutaneous T-cell lymphoma (in collaboration with the Clinical Center Nuclear Medicine Department, NIH/Naval Medical Oncology Branch, and others) produced the most efficient, antigen-specific imaging yet achieved in human subjects. (6) Extension of the lymphatic technique to reach node groups inaccessible from subcutaneous injection sites. In particular, antibodies injected via a bronchoscope into peri-bronchial tissue labeled the pulmonary lymph nodes efficiently and selectively in experimental animals. The

technique is being incorporated into protocols for human lung cancer. (7) Development of kinetic models for the pharmacology of monoclonal antibodies in animals (see Report No. 4) and humans.

Publications:

Weinstein JN, Leserman, LD. Liposomes as drug carriers in cancer chemotherapy In: Goldman, ID, ed. The International Encyclopedia of Pharmacology and Therepeutics. Vol 118. Membrane Transport of Anti-neoplastic Agents. New York: Pergamon, 1986;169-194.

Parker RJ, Weinstein JN, Keenan AM, Dower SK, Steller MA, Holton OD, Sieber SM. Targeting of radiolabelled monoclonal antibodies in the lymphatics. Cancer Research, 1987; 2073-1076.

Weinstein JN, Parker R, Holton OD III, Black CDV, Sieber SM, Covell DC. The pharmacology of monoclonal antibodies. In: Bonavida B, Collier RJ, eds. Membrane-mediated Cytotoxicity, New York: Alan R Liss, 1987;279-289.

Weinstein JN. Immunolymphoscintigraphy and other regional applications of monoclonal antibodies In: Zalutsky M ed. Antibodies in Radiodiagnosis and Therapy, Boca Raton, FL: CRC Press, in press

Mulshine JL, Keenan AM, Carrasquillo JA, Walsh T, Linnoila RI, Holton OD, Harwell J, Larson SM, Bunn PA, Weinstein JN. Immunolymphoscintigraphy of pulmonary and med iastinal lymph nodes. A new approach to lung cancer imaging. Cancer Research, 1987;47:3572-3576.

Covell DG, Barbet J, Holton OD III, Black CVD, Keenan AM, Sieber SM, Weinstein JN. Global and local factors in the pharmacology of monoclonal antibodies. American Statistical Society Symposium, in press

Eger RR, Covell DG, Carrasquillo J, Larson SM, Reynolds J, Abrams P, Morgan, AC Schroff R, Foon K, Weinstein JN. A kinetic model for the biodistribution of an In-111 labeled monoclonal antibody in humans, Cancer Research, 1987;47:3328-3336.

Weinstein JN, Black Christopher DV, Holton OD III, Covell DG, Parker RJ, Mulshine JL, Lotze MT, Carrasquillo J, Eger RR, Lewis A, Larson SM, Keenan AM. Delivery of monoclonal antibodies to lymph nodes via the lymphatics In: Winkelhake JL, Holcenberg JS, eds. The Pharmacology and Toxicology of Proteins, New York: Alan R Liss, 1987;75-89.

Kroczek RA, Black CDV, Barbet J, Shevach EM. Induction of IL-2 expression in vivo. Response to allogeneic cells. Transplantation, 1988, in press.

Kroczek RA, Black, CDV, Barbet J, Shevach EM. Mechanism of action of Cyclosporin A in vivo l Cyclosporin A fails to inhibit T lymphocyte activation in response to alloantigens, J of Immunology, 1988, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08363-06 LTB

PERIOD COVERED: October 1, 1987 to September 30, 1988

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

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

H. Robert Guy, Ph.D.	Senior Staff Fellow	LTB, NCI
Other Professional Personnel:		
G. Raghunathan	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 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The primary goal of this project is to develop methods to predict structures of membrane proteins from their sequences and available experimental data and to use these methods to develop structural models of specific membrane proteins. Most of our effort has been devoted to developing methods of combining computer graphics techniques with energy calculations by the program CHARMM. Structural models were developed for a series of peptides (melittin, δ lysin, magainin, PGLa, and pardaxin) that interact with membranes to lyse cells and/or form membrane channels. We modeled these systems to study how these monomers aggregate on one surface of a membrane, how they insert across the membrane, and how they form channels. All of these peptides have sequences consistent with their forming amphipathic α helices. Melittin and δ lysin are lytic toxins from bee venom and staphylococcus. Magainin, PGLa, and XPF are potent antimicrobial agents secreted from Xeneopus granular glands. They resemble melittin in that they can form positively charged amphipathic α helices; however, they are not hemolytic at concentrations that kill microbes. Magainin induces anion selective channels in artificial membranes. PGLa and XPF are homologous peptides that have no homology to Magainin but have very similar antimicrobial effects. Pardaxin is a potent shark repellent made by the Red Sea Moses Sole. It forms channels in artificial membranes that, unlike most other α helical peptide channels, appear to have only one size. Pardaxin channels allow inorganic cations and anions but not Tris base or large organic anions to pass.

GPO 914-918

Project Description:

Major Findings:

It is important to test new methods of predicting protein structure in a manner that cannot be biased by knowing the structure. We developed a model of δ lysin and how it aggregates in the crystal as a test of our methods for combining computer graphic procedures with energy minimization methods. This peptide has been crystallized but the crystal structure has not yet been determined.

In our models all of the peptides mentioned in the abstract form antiparallel aggregates with hydrophobic side chains on one side of the aggregate and predominately hydrophilic side chains on the opposite side. Planar 'raft' type aggregates are postulated to displace lipids on one membrane surface for all these peptides. Models of cylindrical channels were developed for magainin, PGLa, and pardaxin. These models are consistent with the present data on channel properties in lipid bilayers. The transition from the raft to the channel structure is postulated to occur when two rafts meet 'end-on' and then fold into the membrane in a way that does not expose the hydrophilic side chains to the lipid alkyl chains. This new hypothesis for how α helices can insert across the membrane has important implications for understanding membrane insertion mechanisms of protein and for developing criteria to identify possible transmembrane segments.

We developed a model for the transmembrane portion of GABA and glycine receptor channels by combining methods developed for the peptides with methods we had previously developed for large integral membrane channel proteins. This model has several features similar to our models of the peptides: two of the helices have a series of phenyl rings that pack together in a manner similar to our magainin model; the pore is lined primarily by serine and threonine side chains similar to our pardaxin model, and helix packing postulated to occur between adjacent receptors is very similar to packing we propose for the δ lysin crystals and for pardaxin tetramers in solution.

Sequences of voltage-activated calcium and potassium channels were analyzed and related to our previously developed model of the sodium channel to develop models of calcium and potassium channels.

We also developed a model of the three-dimensional structure of the receptor binding site of Type 3 reovirus.

Publications:

Guy HR, Raghunathan G. Structural models for membrane insertion and channel formation by antiparallel α helical membrane peptides. Transport Through Membranes: Carriers, Channels, and Pumps 21st Jerusalem Symposium on Quantum and Biochemistry. ed. Albete Pullman. In press.

Guy HR, Raghunathan G. Structural models of alpha helical membrane peptides and the GABA receptor channel. Computer-assisted Modeling of Receptor-ligand Interactions: Theoretical Aspects and Applications to Drug Design. 1988 OHOLO Conference. In press.

Guy HR. Molecular Models for Voltage Activated Channels. Proceedings of International Conference on: Molecular and Cellular Mechanisms of Antiarrhythmic Agents. In press.

Guy HR, Hucho F. The ion channel of the nicotinic acetylcholine receptor. Trends in Neuroscience. 1987 10:318-321.

Guy HR. A model relating the sodium channel's structure to its function. For: Molecular Biology of Ion Channels. In: Agnew W, Sigworth, F and Claudio, ". eds. Current Topics in Membrane Transport. In press.

Forte M, Guy HR, Mannella C. Molecular genetics of VDAC ion channel: Structural model and sequence analysis. J. Bioenergetics and Biomembranes. 1987 19:341-350.

Williams WV, Guy HR, Rubin DH, Robey F, Myers JN, Kieber-Emmons T, Weiner DB, Green MI. Sequence of the cell attachment site of reovirus type 3 and modeling of its three-dimensional structure. Proc. Natl. Acad. Sci. USA. In press.

Williams WV, Guy HR, Cohen JA, Weiner DB, Green MI. Molecular and immunologic analyses of a functional internal image formed by an anti-receptor antibody. Annales d' Immunologie. In press.

Williams WV, Guy HR, Cohen JA, Weiner DB, Green MI. Structure and regulation of internal image idiotypes. In: Progress in Allergy. In press.

Williams WV, Moss DA, Weiner DB, Cohen JA, Guy HR, Green MI. Anti-idiotype modeled peptides with biologic activity. Proceedings of the 4th Congress on Immunopharmacology. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08365-06 LTB

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Prediction of T-cell Antigenic Sites from the Primary Sequence

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

James L. Cornette, Ph.D.	Expert	LTB, NCI

Other Professional Personnel

Hanah Margalit, Ph.D.	Visiting Fellow	LTB, NCI
John L. Spouge, M.D., Ph.D.	Visiting Associate	LTB, NCI

COOPERATING UNITS *(if any)*: Jay A. Berzofsky, Metabolism Branch, DCBD, NCI; Charles DeLisi, Dept. of Biomathematical Sciences, Mount Sinai Medical School, New York, NY

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

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.)*

T lymphocytes (T cells) recognize only a small number of segments (antigenic sites) of a foreign protein, and only when the segment is presented on the surface of an antigen presenting cell in association with a major histocompatibility (MHC) molecule. The initial phase of this project is to identify by statistical methods properties of the known antigenic sites that distinguish them from the rest of the protein. Because the available data is mostly for helper T cell sites, the study is limited to these, but some sites specific to cytotoxic T cells are becoming known and the methods developed will be applicable in the study of cytotoxic T cell and eventually antibody sites as well. These methods now include powerful matching techniques from statistical studies of human disease which can assess the significance of individual contributions to antigenicity while accounting for the cross-correlations between putative predictive parameters. Prediction of T-cell sites from the primary sequence will be important in the design of synthetic vaccines.

We continue our study on intrinsic sequence features that are important for T-cell recognition, and pursue the question of MHC restriction. Our recent approach is to simulate the complex of antigen-class II molecule, based on the known structure of the class I molecule. By repeating the analysis for all the antigens that are restricted to a specific MHC, we look for consistent rules, and then test our hypothesis by simulating the same antigens with other MHC types for which they are known not to be restricted. [See also report Z01 CB 04020-11 MET of Jay A. Berzofsky, who, together with Charles DeLisi, initiated many of the questions and with whom this work is closely coordinated.]

This laboratory also collaborated with Attila Szabo and Noam Agmon (LCP, NIDDK) on the analysis of chemical reaction kinetics related to florescence quenching.

GPO 914-918

Project Description

Major Findings:

The first property that was proposed to be characteristic of helper T cell antigenic sites was that the segment, if folded as an α-helix, would form an amphipathic helix, with one side of the helix having hydrophobic residues and the opposite side with hydrophilic residues. This property was tested on 23 sites and found to be a statistically significant characteristic of helper T cell sites, and a predictive algorithm based on this property was written. Previously, the algorithm was used successfully to predict sites in the circumsporozorite protein (CS) of Plasmodium falciparum and the gp120 envelope protein of the AIDS virus HTLV-III [3], as reported. When newly discovered antigenic sites were included in our analysis to expand the data base to 44 sites, the correlation with helical amphipathicity was still highly significant ($p < 0.003$) [1]. Additional predictions have been made and tested. The predictions were successful in the acetyl choline receptor and in the Pf155 protein of the blood stage of the malaria parasite, and were not successful in the interphotoreceptor retinoid-binding protein [5]. A particularly interesting result is that a site predicted by the algorithm in the envelope protein of the human immunodeficiency virus was found to be immunodominant for T cells [8].

Matching statistical techniques were applied to a suitable database of helper T cell antigenic sites and supported the following structural generalities [6]. 1) Helper T cell sites have a tendency to take an α-helical conformation; 2) the resulting α-helices tend to be amphipathic; 3) the sites tend to have lysine close to their COOH-termini; and 4) the sites tend not to be segmentally amphipathic. The novel use of matching techniques in this peptide study shows that these four properties are independent predictive parameters. Work to produce a predictive scheme encompassing these four parameters is now in progress.

From the known three dimensional structure of class I molecule together with conventional sequence comparisons we determined the potential α helical segments of the class II molecule. These segments were modeled by us as having an α-helical configuration using the known coordinates of the class I helices. There are many possible conformations for complexes formed between the two α helices and an antigenic α helix. The likelihoods of each conformation is evaluated so that the most favorable ones can be determined. The simulations are done for each Ia type and the data base of its known antigens separately. From these models, specific sequence features that determine the MHC restriction can be studied and it is hoped that the properties determining immunodominance of antigenic sites can be understood at the molecular level. Understanding the biochemistry of these structures will be valuable for the design of synthetic or recombinant vaccines.

A new method for solving polymerizing and annihilating chemical reactions was also found and applied to give several new analytic solutions for these reactions [7]. Drs. Szabo and Agmon (LCP, NIDDK) are presently using the techniques developed in the analysis of the long-time behavior of fluorescence quenching reactions.

Publications

Berzofsky JA, Cease KB, Cornette JL, Spouge JL, Margalit H, Berkower IJ, Good MF, Miller LH, DeLisi C. Protein antigenic structures recognized by T cells: Potential application to vaccine design. Immunol Reviews 1987;98:9-52.

Cease KB, Margalit H, Cornette JL, Putney SD, Robey WG, Ouyang C, Streicher HZ, Fischinger PJ, Gallo RC, DeLisi C, Berzofsky JA. Helper T cell site identification in the AIDS virus gp120 envelope protein and induction of immunity in mice to the native protein using a 16-residue synthetic peptide. Proc Nat Acad Sci USA. 1987;84:4249-4253.

Spouge JL, Berzofsky JA. Statistics in immunology and biochemistry: An application to T cell antigenic sites. In: Perlson AS, ed. Theoretical immunology, Part Two. Santa Fe: Proc of the Theoretical Immunology Workshop, June 1987, 131-146.

Berzofsky JA, Berkower IJ, Cease KB, Margalit H, Cornette JL, Spouge JL, Spencer C, Buckenmeyer G, Streicher H, Kojima M, DeLisi C. The role of MHC and amphipathic structures in T cell recognition: Features determining immunodominance. In: Vogel H, Pernis B, Silverstein S, eds. Processing and presentation of antigens. New York: Columbia University Press, 1988;125-131.

Spouge JL. Exact solutions for a diffusion reaction process in one dimension. Phys Rev Lett. 1988;60:871-874.

Takahashi H, Cohen J, Hosmalin A, Cease K, Houghten R, Cornette JL, DeLisi C, Moss B, Germain R, Berzofsky J. An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine. Sci USA, 1988;85:3105-3109.

DeLisi C, Cornette JL, Margalit H, Cease K, Spouge J, Berzofsky JA. The role of amphipathicity as an indicator of T-cell antigenic sites on proteins. In: Secarz EE, Berzofsky JW, eds. Immunogeneicity of protein antigens: Repertoire and regulation, Boca Raton: CRC Press, in press.

Sanui H, Redmond TM, Hu L, Kuwabara T, Margalit H, Cornette JL, Wiggert B, Chader GJ, Cery I. Synthetic peptides derived from IRBP induce EAU and EAP in rats. Current Eye Research 1988, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08366-05 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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:		
Kenji Fujimori, M.D.	Visiting Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

Dr. L. Liotta, LP, DCBD; Dr. John Fletcher, LAS, DCRT

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors B

☐ (a2) Interviews

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. 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. (5) Most interesting, antibodies with low affinity may be preferable at a given dose to those with high affinity for some therapeutic applications.

We plan to test predictions of the model using micrometastases of human melanoma in nude mice. 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 lymphokines and growth factors.

GPO 914-918

Project Description

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; (5) Antibodies of low affinity may be preferable to those of high affinity at a given dose for some purposes, e.g. in therapy with alpha-emitting conjugates or toxin conjugates. (6) Quantitative, but not qualitative, changes in predicted behavior are seen as the geometry changes from Cartesian to cylindrical (for cords of tumor cells surrounding a central blood vessel) to spherical$_2$(for flux into a nodule of cells); (7) the characteristics for IgG, F(ab')$_2$, and Fab can been compared; (8) Bivalent binding can be simulated; (9) the global pharmacology can be integrated with the percolation calculations.

Publications:

Weinstein JN, Parker R, Holton OD III, Black CDV, Sieber, SM, Covell DG. The pharmacology of monoclonal antibodies. In Bonavida B, Collier RJ eds. Membrane-mediated Cytotoxicity, New York, Alan R. Liss, 1987;279-289.

Weinstein, JN. Immunolymphoscintigraphy and other regional applications of monoclonal antibodies. In: Zalutsky M ed. Antibodies in Radiodiagnosis and Therapy, Boca Raton, FL, CRC Press, in press.

Weinstein JN, Black CDV, Holton OD III, Covell DG, Parker RJ, Mulshine JL, Lotze MT, Carrasquillo J, Eger RR, Lewis A, Larson SM, Keenan AM. Delivery of monoclonal antibodies to lymph nodes via the lymphatics. In Winkelhake JL, Holcenberg JS eds. The Pharmacology and Toxicology of Proteins, New York, Alan R. Liss, 1987;75-89.

Covell DG, Barbet J, Holton OD III, Black CVD, Keenan AM, Sieber SM, Weinstein JN. Global and local factors in the pharmacology of monoclonal antibodies. American Statistical Society Symposium, in press.

Weinstein JN, Eger RR, Covell DG, Black CDV, Mulshine J, Carrasquillo JA Larson SM, Keenan AM. The pharmacology of monoclonal antibodies. Annals N.Y. Acad. Sci., in press.

Black CDV, Atcher RW, Barbet J, Brechbiel MW, Holton OD III, Hines JJ Gansow OA, Weinstein JN. Selective ablation of B lymphocytes in vivo by an alpha emitter, ^{212}bismuth, chelated to a monoclonal antibody. J. Antibody, Immunoconjugates and Radiopharmaceuticals, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08367-05 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 charecters 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:

John N. Weinstein, M.D., Ph.D., Senior Investigator LTB, NCI
Mary Jane Talley, B.S. Biologist 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

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	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.)*

Following subcutaneous injection, radiolabeled monoclonal antibodies bind efficiently to normal and tumor target cells in the lymph nodes (Project # Z01 CB 08359-03 LTB). This finding prompted us to attempt specific therapy using monoclonal antibody conjugates.

An immunotoxin made from the A-chain of ricin 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. These studies have led to a new hypothesis for the cell biological pathway (the "neutral bypass") by which toxin molecules enter the cytoplasm from antibody conjugates to kill a cell.

A further type of immunoconjugate for specific cell killing in vivo consists of a radioactive compound chelated to an antibody. We have demonstrated selective ablation of lymph node B lymphocytes in mice injected subcutaneously with an anti-murine B cell antibody labeled with the alpha particle emitter ^{212}Bismuth. The relative potency of this conjugate for B cells in vivo was 10-fold higher than for T cells taken from the same nodes.

In order to assess the effects of antibodies and antibody conjugates in vivo, we have established two models of lymph node T cell activation. In one, the stimulus is the plant lectin concanavalin A administered into the footpad; the other stimulus is allogeneic cells. Both of these stimuli induce T cells to express receptors for IL-2. The concanavalin A model is susceptible to the inhibitory effects of cyclosporin A whereas the allogeneic model is not.

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

Project Description:

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. These findings have led to formulation of the "neutral bypass" hypothesis, i.e., the hypothesis that those few molecules of ricin A which reach the cytoplasm to kill a cell do so via a pathway that bypasses the acidified endocytic vesicles of the "classical" receptor-mediated endocytosis pathway.

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): We have made an immonoconjugate by chelating the alpha particle emitter ^{212}Bismuth to a monoclonal antibody through isothiocyanatobenzyl-DTPA. One such conjugate, based on an antibody directed against the murine B lymphocyte determinant LYB8.2, was prepared and used to selectively ablate lymph node B cells in the nodes draining the footpad injection site. At doses which ablated almost all B cells, most of the T cells from the same node remained viable. After treatment, the number of B cells in the node recovered to the pretreatment levels over a period of 48 hours. Following intravenous injection of the same

immunoconjugate, splenic lymphocytes were also ablated although the B cell selectivity was not as high as that seen in the lymph nodes.

5): Immunotoxin experiments demonstrated that lymphocyte killing was much more efficient when the cells were stimulated. In order to optimize immunotoxin killing of lymphocytes in vivo, we have developed two models of in vivo lymphocyte stimulation. In the first, concanavalin A was injected subcutaneously into the footpad. Almost all the lymphocytes from the lymph nodes draining the injection site rapidly expressed the receptor for IL-2 and spontaneously proliferated within 9 hours. In the second model, injection of allogeneic cells caused a smaller number of lymph node lymphocytes to express the IL-2 receptor and to proliferate spontaneously after two to three days. The models induced both B and T cells to express the IL-2 receptor and there was very little difference in receptor expression by either of the two main T cell subsets. Cyclosporin A was able to prevent receptor induction by concanavalin A but not in the allogeneic cell model.

6): Experiments to determine the biological distribution of the uncoupled antibodies following administration via the lymphatic system are described in another report (Project # Z01 CB08359-03 LTB). These experiments must precede the in vivo use of immunoconjugates.

Publications:

Parker RJ, Weinstein, JN, Keenan AM, Dower SK, Steller MA, Holton OD III, Sieber, SM. Targeting of radiolabelled antibodies in the lymphatics. Cancer Research 1987;47:2073-2076.

Weinstein JN. Immunolymphoscintigraphy and other regional applications of monoclonal antibodies. In: Zalutsky M. ed. Antibodies in Radiodiagnosis and Therapy, Boca Raton, FL, CRC Press, 1988, in press.

Mulshine JL, Keenan AM, Carrasquillo JA, Walsh T, Linnoila RI, Holton OD III, Harwell J, Larson SM, Bunn PA, Weinstein JN. Immunolymphoscintigraphy of pulmonary and mediastinal lymph nodes: A new approach to lung cancer imaging. Cancer Research 1987;47:3572.

Covell DG, Barbet J, Holton OD III, Black CDV, Keenan AM, Sieber SM, Weinstein JN. Global and local factors in the pharmacology of monoclonal antibodies. American Statistical Society Symposium, 1988, in press.

Eger RRM Covell DG, Carrasquillo J, Larson SM, Reynolds J, Abrams P, Morgan AC, Schroff R, Foon K, Weinstein JN. A kinetic model for the biodistribution of an In-111 labeled monoclonal antibody in humans. Cancer Research 1987;47:3328-3336.

Weinstein JN, Black CDV, Holton OD III, Covell DG, Parker RJ, Mulshine JL, Lotze MT, Carrasquillo J, Eger RR, Lewis A, Larson SM, Keenan AM, Delivery of monoclonal antibodies to lymph nodes via the lymphatics. In Winkelhake JL, Holcenberg JS, eds. The Pharmacology and Toxicology of Proteins, New York, Alan R. Liss, 1987;75-89.

Kroczek RA, Black CDV, Barbet J, Shevach EM. Induction of IL-2 expression in vivo: Response to allogeneic cells, Transplantation, in press.

Kroczek RA, Black CDV. Barbet J, Shevach EM. Mechanism of action of Cyclosporin A in vivo. 1. Cyclosporin A fails to inhibit T lymphocyte activation in response to alloantigens, J of Immunology, 1988, in press.

Black CDV, Atcher RW, Barbet J, Brechbiel MW, Holton OD III, Hines JJ, Gansow OA, Weinstein JN. Selective ablation of B lymphocytes in vivo by an alpha emitter, ^{212}bismuth, chelated to a monoclonal antibody. J. Antibody, Immunoconjugates and Radiopharmaceuticals, 1988 in press.

Keenan AM, Weinstein JN, Carrasquillo JA, Bunn PA Jr, Reynolds JC, Foon KA, Smarte NC, Ghosh B, Fejka RM, Larson SM, Mulshine JL. Immunolymphoscintigraphy and the dose-dependence of indium-111-labeled T101 monoclonal antibody in patients with cutaneous T-cell lymphoma, Cancer Research 1987;47:6093-6099.

Black CDV, Kroczek RA, Barbet J, Weinstein JN, Shevach EM. Induction of IL-2 receptor expression in vivo: Response to concanavalin A. Cellular Immunol 1988; 111:420-432.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08369-05 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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 Shapiro, Ph.D.,	Computer Specialist	IPS, LTB, NCI
Morton Schultz,	Senior Engineer	IPS, LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Image Processing Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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 space provided.)*

The object of this part of the program is to make available to the general biomedical community the various microscope control and image acquisition/analysis procedures developed within the section. The plan is based on the quantitative microscopist having available to him as a minimum system a PC/AT (or a fully compatible MS-DOS machine) with a minimum of 512 Kb of memory and a standard 30 megabyte hard disk. All final interfacing with the microscope will be accomplished via commercially available interface cards which have the appropriate analog and digital signal I/O. Image acquisition hardware will also be of the commercial type. We plan to place in the public domain a series of source programs, written in C (for maximum portability in the PC world) which will allow incrementally more varied and complex functions to be performed on images acquired under computer microscopic control. The procedures which the user can employ will depend upon how much additional resources he has available beyond the noted minimum. Functionally speaking, the programs for control, will for example, apply equally well to inverted microscopy (opening up tissue cultures to controlled image acquisition). Liberal provision is being made in the software for control of additional stepping motor functions which could be user defined (e.g. condenser focus, flow of liquids into and out of a chamber, etc). It is planned to include the possibility for interactive image processing, employing a PC compatible high resolution display with gray level capability. This and other options are available as a function of user interest and the level of resources available on the particular PC or PCs in question.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Major Findings:

Since all the source code in C is written as a large set of (usually small) routine, user customization of the software to meet the configuration of this microscope, interface and computer facilities, is a very complex matter. This problem is solved in an interim manner by providing a set of batch files for compilation and loading of various options. Subsequently it is planned to provide a "setup" program which will allow user option selection from large treed menus; this is in line with current preferred software practice.

The remodeling of the space in the Park building was completed during the fiscal year. It was not possible to perform actual interfacing either of hardware or software during this period. Developments in processor design, suggested a change in hardware plans. It was decided to upgrade our present 286 machines rather than replacing them by IBM PS/2 's as originally planned. This will permit the retention of all of our interface hardware and software, while allowing a local ethernet configuration based on a small Sun processor (delivery expected before the end of the fiscal year), using the two PCs as nodes. This will have the further advantage of more direct relations to the facility at Frederick allowing much needed networking with the section and lab personnel from whom we are unfortunately physically separated.

A major effort involving BEIB cooperation involves the construction of a generalized microscope hardware interface, which will allow sufficient generality for task specific commercial hardware selection in terms of the particular investigators needs.

At present under consideration is possible operation of the system under OS/2. It is not clear that the IBM system will be very widely used for the next several years, the upgrading to OS /2 and the Presentation Manager involving as it does a quantum leap in hardware and memory resource requirements. This option is therefore of only low priority for the remainder of this and the next fiscal year. Of far greater interest is the possibility of a Xenix or UNIX version. This is in part dependent on the applicability of some commercially available realtime software interface programs. The latter is now being explored.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08370-05 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Interactions in Globular Proteins and Protein Folding

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:

David Covell, Ph.D.	Expert	LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

A new approach has been taken to the protein folding problem. It is a general approach that yields all possible topologies of the protein chain, but not yet all of its atomic details. It affords an enormous reduction in the number of protein conformations that it is necessary to consider for the complete problem. The procedure is to generate all possible compact conformations and to assess their quality in a general way, based upon what has been observed in globular protein crystal structures. This effectively leads to "fuzzy" protein conformations, with only the major features of the residues that are close to one another. We have generated, on cubic lattices, all possible conformations of crambin and trypsin inhibitor. In each case only several thousand conformations are possible in their limited space. For example for crambin this represents a reduction from around 10**30 to about 2000 conformations. To assess the merits of these conformations, we use effective residue-residue interaction energies statistically derived from protein X-ray structures. The native conformations have been observed always to fall in the best 1 to 10% of the conformations, yielding a further reduction of at least an order of magnitude in the number of conformations that must be subsequently considered. We plan to develop procedures to include all atoms and refine with conventional energy calculations, for the best few hundred of these conformations.

Project Description

Major Findings:

A principal goal of molecular biology is to understand the bases of molecular and biological recognition. An ultimate goal for theory in this area remains the calculation of favored macromolecular conformations directly from their sequences. Although we occasionally utilize detailed atom-atom calculations, we feel the development of higher order principles of molecular structure is essential if we are to achieve a complete understanding of all of the complexities of biological macromolecules themselves, as well as their interactions with other small molecules, other macromolecules and their assembly into biological structures. This project is progress in that direction.

We have collected statistics on globular proteins from their X-ray structures, counting the amino acid residues that are frequently found near one another in the three dimensional structures. These were obtained in the following way: a lattice 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. These derived contact energies follow intuition with the most favorably interacting pairs being hydrophobic residues. This type of energy has a direct bearing on folding and substantially reflects the effects of water on folding. There is a segregation of hydrophobic and hydrophilic residues. These values reflect the actual situation inside proteins and provide a tool that can be applied to a variety of problems and, in the problem at hand, can be used to assess the relative overall quality of different conformations.

The present examples of generating all possible compact conformations on dense lattices indicate that it should be possible to generate all compact conformations of any small protein, with one lattice point per amino acid.

Publication:

Jernigan RL, Sarai A, Covell DG, Mazur J. Exhaustive enumeration and evaluation of feasible conformations for double helix DNA and folded proteins. Proceedings of the 1988 Third International Conference on Supercomputing and Second World Supercomputer Exhibition. Boston: International conference on Supercomputing. 1988;1:197-200.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08371-05 LTB

PERIOD COVERED: October 1, 1987 to September 30, 1988

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

Other Professional Personnel:

Akinori Sarai, Ph.D., Visiting Associate, LTB, NCI
Bruce Shapiro, Ph.D., Computer Specialist, IPS, LTB, NCI

COOPERATING UNITS *(if any)*: Dr. Ruth Nussinov, Institute of Molecular Medicine, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; Dr. Jacob Mazur and Dr. David Wang, Program Resources, Inc., Frederick, MD

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	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.)*

To investigate the effects of sequence on double helix DNA conformations we have developed methods to average over intra-helical degrees of freedom to obtain conformational means and fluctuations. This method is general and yields intra-molecular DNA conformational energies appropriate to study many aspects of the sequence dependence of conformations. In this method, each base pair has been permitted to interact with its neighboring base pairs over a large range of conformations, and the average is extracted in an iterative way. Interaction energies have been evaluated with semi-empirical energy functions. Even though the backbone has not been included in these calculations, interactions among the base pairs alone lead to a strong preference for double helical conformations. The best conformation obtained for the dodecamer, d(CGCGAATTCGCG), compared to the crystal structure, has a RMS deviation of 0.8 A. The co-crystal of this Eco RI recognition site with the Eco RI restriction enzyme shows a substantial helical twist deviation at the AT junction; calculations have confirmed this as the site of largest twist fluctuations in the sequence.

GPO 914-918

Project description

Major Findings:

Double helical DNA is highly exposed to solvent, in contrast to residues in a globular protein. Thus it seems apparent that its conformations should be more readily affected by its environment. Conformations of DNA can span a wide range, from the usual Watson-Crick B-form helix to the left handed Z-form. Recently it has become clear that there is a continuum of conformations, within each family of conformations. Specifically there are sequence dependent variations in the vicinity of the B-form. These latter variations are the subject being studied here. It is interesting to consider whether or not these variations are large enough to affect recognition processes.

One of the most interesting results of the present study is that the helical structure of DNA appears to be determined predominantly by interactions among base pairs, with twist angles clustered about 36^{0}. Principally it appears to be the electrostatic energy that stabilizes these double helix twist angle. We observe that opposite charges tend to be particularly favorably arranged in the helical orientation of base pairs. In these calculations there is a partial electric charge on all atoms. Also, the formulation includes an adjustable parameter, namely, the electrostatic shielding constant, to reflect the effects of the sugar-phosphate backbone. This parameter has been treated as a single adjustable parameter.

The sequence dependence of calculated twist, roll, tilt and propeller twist for the dodecamer sequence d(CGCGAATTCGCG) are qualitatively in good agreement with the experimental structure. The roll values agree especially well with experiment. In this calculation, with the backbone atoms absent, the calculated structure of the dodecamer agrees remarkably well with the X-ray structure, giving a RMS deviation of about 0.8. Also we calculated the thermal fluctuation at each base step in terms of the Euler angles, twists, rolls, tilts and propeller twists. The fluctuations depend on the sequence. For example, the smallest fluctuations in twist and roll are at the CG steps; whereas the largest fluctuation in twist is for the AT pair and the largest for roll is at GC pairs. We are also studying sequence-dependent fluctuations more generally.

A look at the reported structures of proteins bound to DNA gives a clue to the sequence dependence of flexibility. In the case of the Eco RI - DNA complex, i.e. the binding to the DNA sequence we have treated here, the largest kink is at the AT junction where it is undertwisted by about 25 degrees. This is qualitatively consistent with our results that indicate this site to be most susceptible to twist fluctuations. The energy dependence on twist of the central junction for the fragment AATT also shows an extremely flat surface. The experimental structure together with these calculations shows that the AT junction is the most flexible and can assume a broad variety of conformations.

A major question about DNA conformations is: To what extent are conformational variations intrinsic to the sequence or induced by protein binding or interactions with other molecules? We have investigated the first aspect of the question and are beginning to study the latter possibilities. For some special sequences, there is evidence for the intrinsic sequence effect from experimental gel electrophoresis in which large bends are indicated in isolated DNA fragments by their slower mobilities.

We are initiating a study to characterize the types of bends that can be readily induced in specific DNA sequences to see what radii and shapes are accessible. Specificity from the sequence could in this way be manifested in such variable shapes. Also in a major extension of the method, all the backbone atoms are now being included in the calculations.

Publications:

Nussinov R, Sarai A, Wang D, Jernigan R L. Sequence context of homooligomer tracts in Eukaryotic genomes: Some DNA conformational implications. In: Olson WK, Sarma MH, Sarma RH, Sundaralingam M, eds. Structure & expression Vol. 3: DNA bending and curvature, NY: Adenine Press, 1988;129-138.

Sarai A, Mazur J, Nussinov R, Jernigan RL. Sequence dependence of DNA conformations: Means and fluctuations. In: Olson WK, Sarma MH, Sarma RH, Sundaralingam M, eds. Structure & expression Vol. 3: DNA bending and curvature, NY: Adenine Press, 1988;213-223.

Mazur J, Sarai A, Jernigan RL. Interaction range in B-form DNA double helices. Proceedings, 12th IMACS World Congress, International Association for Mathematics and Computers in Simulation, Paris, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08372-05 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Molecular Recognition of DNA

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

Akinori Sarai, Ph.D.,	Visiting Associate	LTB, NCI
Other Professional Personnel:		
Robert Jernigan, Ph.D.,	Theoretical Physical Chemist	LTB, NCI

COOPERATING UNITS *(if any)*

Yoshinori Takeda, Ph.D., Program Resources Inc., Frederick, Md.	LTB, NCI

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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 binding of Cro and λ repressor to the synthetic operator DNA has been studied by systematic base substitutions. From this data we deduce specific interactions between Cro and the operator DNA and estimate the energetic contribution of each interaction to the specific binding. Such studies clearly show that the recognition of specific DNA sequences by Cro and λ repressor is mediated by a combination of bi-dentate H-bonds between amino acid side chains and base pairs, and hydrophobic interactions with thymine's methyl groups as exposed within the DNA major groove. Losses of such H-bond or hydrophobic interactions reduce the binding free energy by 0.9 to 3.1 Kcal/mol or 0.8 to 1.8 Kcal/mol, respectively. The free energy changes are principally additive for the specific binding, but not additive for nonspecific binding. These interactions described here are not only the specificity determinants in the sequence recognition, but also provide a large part of the binding free energy for the specific interactions of Cro and λ repressor with DNA. Cro and λ repressor recognize the same operator sequences in quite different ways. Site-directed mutagenesis has been used to further study the interaction between amino acids of cro and base pairs. This study provides valuable information about the nature of the Cro-DNA interaction. Computers have been used to analyze the detailed molecular interactions in the repressor-operator complex, and to assist the site-directed mutagenesis.

GPO 914-918

Project Description:

Major Findings:

We have studied the specific interactions involved in the binding of λ repressor with their operator DNA sequences, by mapping the binding affinity change of the repressor with synthetic operator, OR1, and all possible single mutants. The results demonstrate that: 1) the specific interactions are quite asymmetric with respect to the center of the approximate two-fold symmetry of the operator sequence, in contrast with the Cro binding, 2) the non-α-helix part (N-terminal and loop region between helices) of the binding domain make more contribution than the α-helix to the specific interactions, also in contrast to Cro, 3) the specific interactions are due to a combination of hydrogen bonds and hydrophobic interactions between side chains and base pairs. Their contributions in free energy change are as large as 3.7 and 2.0 Kcal/mol, respectively, 4) we have proposed a detailed molecular model of the specific interactions involved in the λ repressor-operator complex based on the results and computer modeling of the repressor-operator complex., and compared it with the model of Cro-operator complex reported previously, 5) free energy changes are mostly additive among different base substitutions. This result enables us to predict relative binding affinity of the repressor to any DNA sequences. The binding order of the repressor to six α operators predicted from the free energy data indeed agrees with actual measurement.

The network of specific H-bonds and hydrophobic interactions between side chains and base pairs enables Cro and λ repressor to recognize different DNA sequences. It explains how Cro and λ repressor distinguish among different operator sequences. Interactions of DNA with repressor proteins are being studied. The aim is to determine the dependence of the interactions on the DNA sequence and conformation. Molecular modelling has been combined with binding measurements to derive experimentally based interaction energies for the Cro repressor-λ operator system. The data are fit in a highly successful quantitative way. This is the first known attempt to derive detailed interaction energies from binding experiments.

By site-directed mutagensis, we have been able to change the sequence specificity of cro, and identify the amino acids interacting with particular base pairs in the binding complex.

Publication:

Sarai A, Takeda Y. How Cro and λ repressor recognize operator DNA sequences. Protein structure, folding, and design 1987;2:57-64.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08374-04 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Other Professional Personnel:

Kazushi Fujimoto, Ph.D, Visiting Fellow LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, FCRF, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We applied fracture-flip to study the ultrastructure of the membrane surface at the sites of the tight junction strand. In addition, we performed conventional freeze-fracture studies of the morphology of tight junction as seen in side views of freeze-fractured epithelial cells.

Project Description:

Major Findings:

It is our objective to understand how membrane lipids and membrane proteins are organized in regions of intercellular contact (junctions). We postulate that intercellular junctions represent instances of where the geometric topology of the adjoining membranes is distinct from the bilayer continuum interrupted by integral membrane proteins that characterizes undifferentiated portions of the membrane. We used fracture-flip to examine the ultrastructure of the cytoplasmic surface of tight junctions. Proximal jejunum was excised from adult Sprague-Dawley rats under light anaesthesia, and rinsed with cooled Hanks' balanced salt solution. Tissue was incubated for 15-30 minutes in the same buffer at 37°C. During this incubation, new tight junction strands form along the entire length of the lateral plasma membranes of adjacent epithelial cells. The mucosal surface was scraped gently with a scalpel to free the mucosa from the muscularis layer. The epithelial cells were isolated mechanically by squeezing the mucosa through a 18-gauge needle. The isolated cells were fixed with 2% glutaraldehyde for 1 hour at 4°C. After fixation, the specimens were rinsed in phosphate buffered saline, impregnated in 30% glycerol, placed on gold specimen carriers, frozen in partially solidified liquid nitrogen, and fracture-flipped. The isolating process would be expected to cause the components comprising the tight junctional membrane to remove from one lateral membrane and remain attached to other, so the fracture-flip can be performed to reveal the true cytoplasmic surface of tight junctions. On the cytoplasmic surfaces of tight junction the strands were discontinuous and particulated. We plan now the cytochemical characterization of the putative components that may be exposed at the cytoplasmic domain of the tight junction, in particular the localization of ZO-1 a peripheral membrane protein (collab with Dr. D. A. Goodenough, Dept. Anat. School of Medicine, Harvard Univ.)

In general, freeze-fracture exposes tight junctional areas on either the P or the E-face of the lateral plasma membranes of epithelial cells. However, much less is known about the apical views of the tight junction. We freeze-fractured stretched toad bladder epithelial cells to reveal the apical views of the tight junction. Bladders were removed from pithed toads (Buto marinus) and each of the two lobes, hemibladder, was mounted as a sac at the tip of a canula. To stretch the epithelial cells the hemibladders were filled with amphibian Ringer's solution to a maximum volume, and then immersed in 2% glutaraldehyde in Ringer's solution for 2 hour at room temperature. Fixed specimens were conventionally freeze-fractured and shadowed. In the majority of cases, the clevage plane exposed junctional areas of lateral cell membranes, on either the P or the E face. As a result, tight junctional strands were consistently confined to the P-faces, and their complementary grooves on the E-faces were devoid of either particles or strands. In some cases, however, the fracture plane exposed P-faces of apical membranes of adjacent cells. We have now collected stereological documentation which will be analysed and prepared for publication within the next few months.

Publications:

Pinto da Silva P. Geometric topology of membrane fusion: from secretion to intercellular junctions. In: Ohki S. ed. : The International Symposium on Molecular Mechanisms of Membrane Fusion. Rochester: Plenum Publishing Corporation, 1987, in Press.

Pinto da Silva P. Topology, dynamics and molecular cytochemistry of integral membrane proteins: A freeze-fracture view. In: Harris, J.R. ed. Electron Microscopy of Proteins. London: Academic Press, 1987;3:2-38.

Pinto da Silva P. A nonaveraged view of biomembranes: The freeze-fracture image. In Aloia R. C. Ed. Advances in Membrane Fluidity. NY: Alan R. Liss, Inc., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08376-04 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Fracture-Label

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)*

Dept. of Renal and Vascular Pathology, Broussais Hosp. (INSERM/ Unit 28), Paris, France (J. Chevalier, D. Appai, J. Bariety); Institute of General Pathology, Univ. of Rome School of Med., Rome, Italy (M.R. Torrisi, A. Pavan).

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, FCRF, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.1	.1	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We used preparative freeze-fracture to gain cytochemical access to the plasma membranes and extracellular matrices (basement membrane, mesangial matrix) of rat kidney glomeruli. In addition to its proven capacity to locate transmembrane proteins, to follow intracellular traffic of membrane proteins, and to characterize both extracellular and cytoplasmic matrices, the development of the modes of application of fracture-label led to the discovery of an elective mode for storage of tissue biopsies for future cytochemical study. The sequence that we propose, based on our fracture-label studies since 1980 involves chemical fixation, gradual impregnation in 25% (v/v) glycerol followed by rapid freezing in the liquid phase of partially solidified Freon 22 (-156°C) cooled by liquid introgen. We showed that these specimens, which can be stored indefinitely in liquid introgen, can be thawed without loss of ultrastructional detail or of antigenic activity. This sequence allows for the collection of tissue biopsies over extended periods with the cytochemical labeling done in a single experiment once a sufficient number and diversity of specimens have been collected.

GPO 914-918

Project Description:

Major Findings:

During the past year we completed a project on the localization of lectin receptors (concanavalin A; wheat germ agglutinin) and cationic sites on the plasma membranes and extracellular matrices of rat kidney glomeruli. In this project freeze-fracture of tissues was used to gain cytochemical access to the complex, multi-barrier system that characterizes the glomerulus. Previous cytochemical approaches rely on the perfusion of tracers and therefore are limited by the characteristics of the barriers that must be crossed until the label can reach the membrane to be labelled. Fracture of frozen tissues leads to random exposure of membranes as well as of intracellular and extracellular matrices. The fragments can, after thawing, be exposed to cytochemical labeling procedures and then processed for this section microscopy. Our studies demonstrate the feasibility of using glutaraldehyde fixed, glycerol impregnated frozen tissue. The advantages for ultrastructural pathology studies are obvious: tissues can be collected and, after chemical fixation, glycerination and rapid freezing in liquid freon (temp - 156°C) cooled by liquid introgen, can be stored indefinitely. This allows specimen collection and storage over a period of years. When enough specimens (e.g. biopsies) have been collected they can be cytochemically studied "en bloc". This is important because it allows standardization of labeling conditions.

In other studies, conducted at the Institute of General Pathology, University of Rome School of Medicine (M. R. Torrisi; A. Pavan) we used fracture-label to show that, on fracture, CD3 antigens partition preferentially with the inner half of the membrane suggesting that this transmembrane glycoprotein is associated to components of the membrane skeleton and/or the cytoskeleton. At present, the main applications of fracture-label have been illustrated. At present, the main applications of fracture-label have been illustrated: in particular, the identification localization and partition of transmembrane proteins; the following of intracellular traffic of membrane proteins; and the localization of specific sites over extracellular as well as cytoplasmic matrices. The method, discovered in 1981, led also to the discovery that, as stated above, rapid freezing and thawing of chemically fixed, cryoproteded tissues preserves cell ultrastructure and antigenicity. At present, and because of the development and affliction of fracture-flip methods (see our new project), we are using fracture-label only to answer specific questions that may be raised in connection with other projects. Over the next year we expect to prepare for publication the our findings on the cytochemical labeling in autonomic ganglia, primary sensory ganglia and peripheral nerves.

Publications:

Pinto da Silva P. Freeze-fracture cytochemistry: A poor person's guide. Bull Electr Microsc Soc Am 1986;16:113-117.

Chevalier J, Appay MD, Wang XY, Bariety J, and Pinto da Silva P. Freeze-fracture cytochemistry of rat glomerular capillary tuft. Determination of wheat germ agglutinin binding sites and localization of anionic charges. J Histochem Cytochem 1987;35:1389-1399.

Pinto da Silva P. Molecular cytochemistry of freeze-fractured cells: Freeze-etching, fracture label, fracture-permeation and label-fracture. In: Miller KR, ed. Advances in cell Biology. Greenwich: JAI Press, 1987;1:157-190.

Pinto da Silva P. Visual thinking of biological membranes: From freeze-etching to label-fracture. In: Verkleij, A. ed. Immunogold Labeling Methods. Boca Raton: CRC Press, in press, 1988.

Torrisi MR, Pavan A, and Pinto da Silva P. Freeze-fracture cytochemistry of transmembrane proteins in human lymphocytes. EEO J Immunol Immunopharm. in press.

Pinto da Silva P. Freeze-fracture images: 1967-1971. In: Hui SW, ed. Freeze-fracture studies of membranes. Boca Raton: CRC Press Inc., in press.

Pinto da Silva P. A non-averaged view of biomembranes: The freeze-fracture image. In: Aloia R. ed. Advances in Membrane Fluidity, Vol. 3, NY: Alan R. Liss, Inc., in press.

Torrisi MR, Mancini P, and Pinto da Silva P. Cytochemical access to plasma and intracellular membranes of freeze-fractured hepatocytes and salivary gland cells. In: Motta P, ed. Ultrastructural pathology. Italy: in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08377-04 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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. Expert 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 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	0.0

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 examined the role of myoglobin to facilitate oxygen diffusion to active mitochondria in skeletal muscle by constructing computer simulation experiments. Steady state mitochondrial oxygen consumption under different conditions of supply PO_2's in a system with and without myoglobin were examined for a one-dimensional slab of tissue. Oxygen consumption by mitochondria was saturable with the mitochondria located in bands at uniform intervals throughout the tissue. Under these conditions, myoglobin provides a measurable increase in oxygen transport for supply PO_2's below 10 torr and diffusion lengths expected for skeletal muscle fibers. We conclude that only under circumstances where hypoxia lowers PO_2 below 10 torr does myoglobin begin to provide a measurable increase in oxygen delivery to mitochondria.

GPO 914-918

Project Description

Major Findings:

When examined for a mathematical model that is more consistent with muscle histology, that is, where bands of oxygen consumption are spaced at uniform intervals throughout the tissue, the role of myoglobin becomes clearer. Under these conditions myoglobin provides a measurable increase in oxygen transport for PO_2's below 10 torr at the surface and for diffusion lengths expected for skeletal muscle fibers. These results a) illustrate the importance of myoglobin for oxygen delivery to active skeletal muscle during conditions of hypoxia, and b) define conditions where oxygen delivery via free or myoglobin-facilitated diffusion begins to limit the normal function of tissue. This information is important for understanding the role of oxygen supply in the growth and development of normal and tumor tissues and for designing therapeutic strategies for the treatment of tumors and of hypoxia-damaged tissues.

Publications:

Covell DG, Jacquez JA. Does myoglobin contribute significantly to the diffusion of oxygen in red skeletal muscle? Am J Physiology 252 (Regulatory Integrative Comp Physiol) 1987;R341-R347.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08378-04 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Label-Fracture

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:

Catarina Andersson Forsman, M.D., Ph.D Visiting Fellow LTB, NCI
Kazushi Fujimoto, Ph.D. Visiting Associate LTB, NCI

COOPERATING UNITS *(if any)* Dept. of Renal and Vascular Pathology, Broussais Hosp. (INSERM/ Unit 28), Paris, France (J. Chevalier, D. Appai, J. Bariety); Institute of General Pathology, Univ. of Rome School of Med., Rome, Italy (M.R. Torrisi, A. Pavan); Lab. Molecular Biol. (Dr M.C. Willingham).

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, FCRF, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0

CHECK APPROPRIATE BOX(ES)

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

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

We continued the development and application of label-fracture cytochemistry. With label-fracture, cells fixed or unfixed are labeled and then freeze-fractured. Label-fracture allows the observation - in a single, coincident image - the distribution of surface receptors and antigens superimposed on conventional Pt/C casts of freeze-fractured membranes. The resolution (5nm attainable) approaches the molecular level. This year we developed a new method for Label-Fracture: "Replica-Staining Label-Fracture". With this method we show that cytochemical labeling of cell surfaces can be performed by direct, post-fracture staining of freeze-fracture replicas. The new method for label-fracture leads to miniaturization of labeling procedures, allows standardization of labeling conditions and the simultaneous processing of different specimens. The work on localization of CD3 and CD4 antigens in human lymphocytes was continued using fracture-flip (see under Fracture-Flip). In other studies we used label-fracture to locate a glycoprotein related to the multi drug resistance of certain cell lines. We used a monoclonal antibody (MRK-16) in expressive and non-expressive fibroblast cell-lines to locate this glycoprotein (with Dr M. C. Willingham).

Project Description:

Major Findings:

Label-fracture is a new molecular cytochemical approach to map, at a resolution better than 10 nanometers, the position of antigens and receptors on the surfaces of biological membranes. Unlike other cytochemical approaches, with label-fracture a high resolution image of platinum-carbon cast of a freeze-fractured membrane is directly related to the distribution of colloidal gold markers of antigens and receptors. As the intramembrane particles visible in freeze-fracture replicas are taken to represent the sites of integral membrane proteins, it becomes possible to relate the distribution of surface sites to that of structural components of the membrane. We have applied this new method to a variety of cells, receptors and lectin binding sites.

Over the past year we developed a new method—Replica-Staining-Label-Fracture. Replica-staining label-fracture labeling of surface antigens and other receptors is made after fracture and thawing of biological specimens. As the exoplasmic halves of freeze-fracture plasma membranes are stabilized by, and remain attached to their Pt/C replicas we have shown that it is possible to label the antigens and receptors after freeze-fracture and thawing. To achieve this, Pt/C replicas with their attached membrane halves are sequentially floated in primary antibody solutions, washed and labeled either with a colloidal gold conjugated secondary antibody or protein A conjugated to colloidal gold. Human erythrocytes and lymphocytes were used to localize lectin binding sites and a membrane bound antigen on the erythrocytes. Human lymphocytes capped or not were incubated in rabbit anti-human IgG antibody. Colloidal gold sheres found over the fracture-faces but also over the intervening Pt/C cast between exoplasmic fracture faces. However, this adherence of markers to the intervening Pt/C cast is unrelated to the cytochemical labeling of cell surface components as shown in control experiments. This non-specific adsorption can be minimized by adding high salt (500mM NaCl), wetting agents (Tween) or by diluting the colloidal gold solution.

The new variant of label-fracture will standardize labeling conditions, permit simultaneous labeling of different specimens, and allow remarkable miniaturization of label procedures.

Publications:

Kan FWK, and Pinto da Silva P. Molecular demarcation of surface domains as established by label-fracture cytochemistry of boar spermatozoa. J Histochem Cytochem, 1987;35:1069-1078.

Pinto da Silva P. Visual thinking of biological membranes: from freeze- etching to label-fracture. In: Verkleij A ed. Immunogold Labeling Methods. Boca Raton: CRC Press, in press, 1988.

Pinto da Silva P. A non-averaged view of biomembrances: The freeze-fracture image. In: Aloia R ed. Advances in Membrane Fluidity, vol. 3. NY: Alan R. Liss, Inc. In press.

Andersson-Forsman C, Pinto da Silva P. Label-fracture of cell surfaces by replica-staining. J Histochem Cytom, in press, 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08379-04 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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. Expert LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Alfred Yergey, Lab. Theoretical & Phys. Biology, NICHD

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors B

☐ (a2) Interviews

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 calcium 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.

Project Description:

Major Findings:

Significant findings have been made with respect to calcium absorption as a function of age and with respect to estimating the distribution of total body calcium from kinetic measurements. Women beyond the age of 45 demonstrate a significantly slower rate of absorption of dietary calcium than younger women. Premature infants have a significantly faster rate of absorption of dietary calcium when compared to adolescents. The affect of these findings on total body calcium metabolism have yet to be determined. We hypothesize that gut motility may play an important role in determining the amount of calcium absorbed from dietary sources. In particular, the slower absorption of dietary calcium in older women may signal the beginnings of osteoporosis. The compartmental model developed from our data indicates that the internal distribution of total body calcium can be estimated from measurements of blood calcium and urinary calcium. This approach may serve as a relatively simple method for clinically predicting the onset of disorders of calcium metabolism. 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. These studies advance the understanding of total body calcium metabolism in adult women and may lead to a method of advance warning for the onset of osteoporosis and improved methods of maintaining calcium balance in premature infants.

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:

Hillman L, Tack E, Covell DG, Vieira NE, Yergey AL. Measurement of true calcium absorption in premature infants using intravenous ^{46}Ca and oral ^{44}Ca. Pediatric Research, 1988;23:589-594.

Yergey AL, Covell DG, Vieira NE. Direct measurement of dietary fractional calcium absorption using calcium isotopes. Biomedical and Environmental Mass Spectrometry, 1987;14:603-607.

Foster DM, Covell DG, Berman M. Applications of a general method for deconvolution using compartmental analysis. Computer Programs in Biology and Medicine, 1988;18(4):32-52.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08380-04 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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, LTB, NCI

Other Professional Personnel:

Devjani Chatterjee, Ph.D.	Visiting Associate	LTB, NCI
John Owens	Computer Specialist	LTB, NCI
Ruth Nussinov, Ph.D.	Consultant	LTB, NCI
Lewis Lipkin, M.D.	Chief, Image Processing Section	LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

Viral systems are used to model the complex macromolecular processes and organization of normal, infected and transformed cells. Computers are used to study the nucleic acid and protein sequences that embody the information necessary for these systems to function.

Picornaviruses cause diseases typified by polio, colds, hepatitis, and foot-and-mouth diseases. Gene and protein sequences of these viruses are comparatively studied for relationships among them and to other known and hypothetical proteins. Secondary structures of the RNAs are found to vary in correlation with pathological and sequence differences.

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.

Computer analyses of proteins and nucleic acids have been developed and implemented in conjunction with techniques of biochemistry, virology, and electron microscopy on sequences of picornaviruses, adenoviruses, human immunodeficiency viruses. 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. Computer programs are developed locally and elsewhere for application on Cray XMP, VAX and graphic workstations to perform sequence analysis and structure predictions. Methods to assess the significance of predictions use Monte Carlo simulations, evolutionary comparisons and biochemical data. Roles for genes and proteins are deduced by comparison with databases of sequences of known function and structure.

GPO 914-918

Project Description:

Other Professional Personnel (Continued):

Bruce Shapiro, Ph.D.	Computer Specialist	LTB, NCI
Andrzej Konopka, Ph.D.	Visiting Fellow	LTB, NCI
Shu-Yun Le, M.S.	Visiting Fellow	LTB, NCI
Anne Barber, M.D.	Sr. Staff Fellow	LTB, NCI

Major Findings:

Multiple sequence alignment for the detection of signals: Multiple sequences for related genes provide a powerful data set for detection of structural entities that are involved in recognition by enzymes or regulatory elements, and for understanding common structural motifs. A dynamic program, ANA, for creating alignments of hundreds of sequences, extracting the features and assessing the statistical significance of such alignments has been developed on a Silicon graphics workstation using the C language. Extensions to this program permit rapid assessment of the probability of chance occurrence for a given feature using a method based on binomial probability theory. A similar but non-dynamic program has been developed from the existing FEATUR program in pascal on the VAX computer. Using these programs a number of observations of consensus signals in the vicinity of splice sites, promoter sites and other items contained in the GENBANK "feature" table have been compiled. Analogous programs have been developed for use with amino acid sequences. They are used to find common structural domains between HIV proteins and known families of enzymes and structural proteins, other viral sequences and potential antigenic sites.

Analysis of the spatial relationships between subsequences in nucleic acids: Experimental evidence indicates that specific functions of nucleic acid are rarely localized in single, contiguous sequence motifs. The patterns responsible for biological function are often non-contiguous and consist of short oligonucleotides separated by gaps of variable length and composition. A powerful method to detect non-contiguous patterns in large collections of sequences has been developed and named "distance analysis". A program (DISTAN) was written in Fortran 77 on the VAX and Cray-Fortran on the Cray XMP/24. It detects statistically significant non-random spacings between all theoretically possible short oligonucleotides, or oligopeptides in the case of proteins. This method was applied to an extensive study of bacterial and eukaryotic mRNA and gene sequences. It showed that introns are highly non-random sequences with two-base periodicity and strong clustering of dinucleotides. In addition it detected novel three-base periodic patterns in exons and bacterial genes. The method is now being adapted to map domains in nucleic acids and proteins. Thus far it has shown that non-contiguous patterns can be used for correct discrimination between known extracellular and cytoplasmic domains of receptors. Further work will aim at discovery of new function- associated sequence patterns and the development of corresponding mapping procedures.

Homology studies: Sequence comparison is one of the most powerful techniques for understanding the organization and function of biological systems. Medline 1975-1977 has 185 references to sequence and homology studies. In the period from 1986-present it has 2577 references. As the availability of sequence information increases it is likely that such studies will be performed more frequently. There will be a continuing need for methods to compare sequences both more easily and more thoroughly. Knowledge bases of experience with the meaning and interpretation of results will also be needed. A variety of methods are used in the laboratory. One of the most powerful of these uses the Cray XMP to search for regions of local homology with the thorough program, SEQHP, or the more rapid SEQFP/SEQFT programs of Kanehisa, and then to uses the SEQDP program to assess the non-randomness of the matches. These have now been combined and modified to produce output suitable for direct input into relational database programs such as FRAMIS or INGRES. In this way the open reading frames of HIV-related viruses are being examined. As yet it has not been possible to find a very strong homology with any known proteins, but weak homologies are being examined. Weak homology with T antigens of polyoma, SV40 and adenovirus types 2,7 and 12 are observed. Low homologies can not be related clearly to common evolutionary background, and may reflect common structural motif or coincidence. Instances of relationships that are obvious in 3-D structure but are difficult by homology (e.g. the two halves of the enzyme rhodanese), may serve as test cases for methods to assess significance.

Detection of potential RNA secondary structure: RNA and DNA structure are of increasing concern as the innate catalytic activity of nucleotides is being recognized and more experience with protein-nucleic acid interactions is accumulated. Evidence from simple systems suggest that there is a hierarchical precedence to the determination of single-stranded polynucleotide structure in the order that primary sequence determines regions of helical base pairing and stacking that pack into 3-dimensional structures. Thus it is necessary to make reasonable predictions of the secondary structure in preparation to predicting the full structure. Methods have been developed along two lines to assess the secondary structure-forming potential of RNA sequences. In one case alternate structures are explored either by examining sub-optimally folded structures of nearly the same stability as the best ones, and in another method the non-randomness of the structures are examined by comparing the natural sequence with those of the same composition but of varying degrees of random order. Both methods yield agreeable results with experimental data, when it is available. A third method utilizes comparison of structures that appear to be conserved across phyllogenetic or mutational challenge. This latter method is widely accepted but has never been assessed by statistical tests. Using all these methods poliovirus, oncornavirus and HIV-related RNA's, and adenovirus single-stranded DNA are being examined. The 5'-region of a number of picornavirus RNA's have been found to have conserved structures even though there are many base changes. This is due to compensatory changes that preserve the predicted secondary structure. The same regions show predicted stability significantly greater than random sequences of the same composition. The same method finds regions of both greater and less than random stability in HIV sequences. Regions of significant non-stability, or open character, are correlated with the hyper-variable regions of the envelope glycoprotein gene. The method has been improved in efficiency up to 100-fold by an extensive study

to replace the time-consuming Monte Carlo procedure with a lookup table using parameters derived from the base composition. In future it will be possible to extend the method to large numbers of sequences, and to provide the capability to use smaller computers for selected sequences.

Publications:

Hausheer FH, Colvin OM, Barnett G, Baylin S, Crum CL, Maizel JV Jr. Multidisciplinary analysis and design of anticancer drugs in the supercomputer environment. Third International Symposium - Science and Engineering on Cray Supercomputers. Minneapolis: 1987;1:449-452.

Konopka AK, Smythers GW. DISTAN - a program which detects significant distances between short oligonucleotides. Computer Applications in Biosciences 1987;3:193-201.

Konopka AK, Smythers GW, Owens J, Maizel JV Jr. Distance analysis helps to establish characteristic motifs in intron sequences. Gene Anal. Tech 1987;4:63-74.

Le S-Y, Currey KM, Nussinov R, Maizel JV Jr. Studies of frequently recurring substructures in human alpha-like globin mRNA precursors. Computers and Biomedical Research 1987;20:563-582.

Maizel JV Jr. Supercomputing in biomedical research. Proceedings of the Second International Conference on Supercomputing: Industrial Supercomputer Applications and Computations. St. Petersburg: Internt'l. Supercomputing Institute, Inc., 1987;344-347.

Hausheer F, Barnett G, Colvin M, Maizel JV Jr. Analysis and prospective design of drugs for the treatment of cancer and AIDS by supercomputer. Presentation at 1988 Third International Conference on Supercomputing and Second World Supercomputer Exhibition. Boston: International Conference on Supercomputing 1988;1:201-206.

Konopka AK.: Compilation of DNA strand exchange sites for non- homologous recombination in somatic cells. Nucleic Acids Research 1988;15:1739-1758.

Le S-Y, Chen K-H, Braun MJ, Gonda MA, Maizel JV Jr. Stability of RNA stem-loop structure and distribution of non-random structure in the human immunodeficiency virus (HIV-1). Nucleic Acids Research 1988;16:5153-5168.

Le S-Y, Chen J-H, Currey KM, Maizel JV Jr. A program for predicting significant RNA secondary structures. Comp Applications in the Biosci. 1988;4:153-159.

Konopka AK, Chatterjee D. Distance analysis and sequence properties of functional domains in nucleic acids and proteins. Gene Anal Techn. In press.

Le S-Y, Chen K-H, Nussinov R, Maizel JV Jr. An improved secondary structure computation method and its application to intervening sequences in the human alpha-like globin mRNA precursors. Computers Application in the Biosciences. In press.

Le S-Y, Nussinov R, Maizel JV Jr. Studies of local stability in histone, U-SnRNA and globin precursor mRNAs around transcription termination sites. Advances in Mathematics and Computers in Medicine. In press.

Nakata K, Maizel JV Jr.: Prediction of DNA-binding protein by discriminant analysis. Computer Applications in the Biosciences. In press.

Nakata K, Kanehisa M, Maizel JV Jr. Discriminant analysis of promoter regions in E. Coli sequences. CABIOS. In press.

Nussinov R, Barber A, Maizel JV Jr. The distribution of nucleotides near bacterial transcription initiation and termination sites show distinct signals that may affect DNA geometry. J Mol Evolution 1987;26:187-197.

Owens J, Chatterjee D, Nussinov R, Konopka AK, Maizel JV Jr. A fixed-point alignment technique for detection of recurrent and common sequence motifs associated with biological features. Computer Applications in the Bioscience 1988;4:73-77.

Owens J, Chatterjee D, Nussinov R, Konopka AK, Maizel JV Jr. A fixed-point alignment technique for detection of recurrent and common sequence motifs associated with biological feature. Computer Application in the Bioscience 1988;4:73-79.

Rivera VM, Welsh JD, Maizel JV Jr. Comparative sequence analysis of the 5' noncoding region of the enteroviruses and rhinoviruses. Virology in press.

Shapiro B, Nussinov R, Lipkin L, Maizel JV Jr. An interactive dot matrix system for locating significant features in nucleic acids molecules. J Biomolec Str and Dyn 1987;4:697-706.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08381-05 LTB

PERIOD COVERED: October 1, 1987 to September 30, 1988

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)*

Peter F. Lemkin, Ph.D.	Computer Specialist	IPS, LTB, NCI
Other Professional Personnel:		
Lewis L. Lipkin, M.D.	Chief, Image Processing Sect.	LTB, NCI
Morton Schultz	Senior Engineer	IPS, LTB, NCI

COOPERATING UNITS *(if any)*: Dr. Eric Lester, Univ. Tenn. Ctr. for Health Services; Dr. Peter Sonderegger, Univ. Zurich; Dr. Heinz Busse, Univ. of Kiel, W.G, Dr. Michael Alley, NCI/FCRF; Dr. Robert Paul, Univ. of Hawaii.

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Image Processing Section

INSTITUTE AND LOCATION: NCI/Frederick Cancer Research Facility, Frederick, MD 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 B

☐ (a2) Interviews

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

Continuing development of a portable GELLAB-II system. GELLAB is a computer based system for the analysis of sets of 2D electrophoretic gels. GELLAB incorporates sophisticated subsystems for image acquisition and processing, data base manipulation and graphics as well as statistical analysis. It has been applied to a variety of experimental systems in which quantitative and qualitative changes in one or more proteins among hundreds or thousands of unaltered proteins is the basic analytic problem. Keeping track of changes detected using these statistical methods is also a major attribute of the system. The same gel DB may be 'viewed' differently and other statistical differences elucidated. Results can be presented in a variety of tables, plots or derived images. Substantive applications include: analyzing sets of gels from adult human leukemias to identify potential marker proteins for possible microsequencing using a gas-phase sequenator (Lester) as well as axonal proteins synthesized during axonal regeneration (Sonderegger). The objective of creating an exportable version of GELLAB is being actively pursued. The new system, GELLAB-II, will run under UNIX and use X-windows for portable interactive graphics. Later it will be exported to VMS and CRAY systems.

Much of the time this past year was spent converting GELLAB-I software from the TOPS10 environment to the GELLAB-II UNIX environment. This included new UNIX software necessary to fill in the gaps for the SUN and microVAX hardware used with the UNIX system. A major conversion effort from 36- to 32-bit hardware systems included data validation studies to ensure data integrity under the new 32-bit hardware and software.

Project Description:

Major Findings:

Conversion of GELLAB to run on workstations: PSAIL been used extensively since July 1987 for the conversion of GELLAB-I SAIL programs to the GELLAB-II C version. Additional SAIL software (also translated to C) was required and has been written to emulate capabilities present on the DEC10 but missing from UNIX. In addition, a number of the SAIL procedures used in the different GELLAB programs were consolidated into common library modules to facilitate uniformity and minimize debugging required in C. These were also translated to UNIX. Additional library procedures were written to emulate some of the SAIL/DEC10 built-in libraries not normally present in UNIX. These libraries and subsystems could be used in other UNIX projects besides GELLAB-II.

All 22 GELLAB programs (about 70000 lines of original code) have been translated from SAIL to C and are in different stages of completion in terms of additional editing and debugging required in UNIX. The GELLAB-I data base program CGELP has undergone major revision going from 3 large modules to 16 smaller ones in the new CGELP2 version. Some of this effort being required for the conversion, other to simplify the code for eventual maintenance by others, and other changes being enhancements which will make the system easier to use (such as MENUs, history, etc) when finally converted to UNIX.

In order to maximize the ability to interface GELLAB-II with primary data from other 2D gel systems (both academic and commercial systems), some changes were made to the fundamental data structures to allow us to input paired spot CGELP2 data from non-GELLAB sources. This would also allow us to use the statistics and search record- keeping part of GELLAB on other types of biomedical data.

A Portable Picture System: Xpix - a (portable) menu driven image processing system for Xwindows which currently runs on SUNs, microVAXs, etc. under UNIX. Xpix has been used to interactively view and landmark 2D gels (Lester). Xpix has additional capability which makes it suitable for analyzing 1D gels. It was designed so that new functionality can be added through external UNIX programs rather than in making time consuming and more difficult changes to the program itself.

Stabilization of PSAIL SAIL to C translator: The PSAIL project was conceived for the purpose of converting the GELLAB set of programs written in SAIL (which runs on the DEC10) to C (which runs under UNIX). PSAIL has become sufficiently stable so that it was frozen in July, 1987. At this point it is capable of compiling over 99% of our SAIL programs into C - although some minor translation problems still exist. PSAIL has been exported to several other DEC10 systems. Several companies have expressed interest in using PSAIL on their computers both for the original plan (of a portable SAIL compiler) and for conversion of SAIL (or MAINSAIL) code to C.

Collaborations: Additional collaborative work with Dr. Peter Sonderegger at Univ. of Zurich has continued in analyzing secreted axonal proteins. In addition to searching for significant protein differences, the quantitative analysis performed with GELLAB was used to help determine whether these differences were due to diffusion from the central chamber or were actually secreted. A new collaboration was started with Dr. Sonderegger's group to aid in converting the UNIX C version of GELLAB to run on their VMS/uVAX system.

Additional collaborative work with Dr. Eric Lester at Univ. Tenn. Ctr. for Health Services on adult human leukemias and and HL60 cell line has continued with additional analysis of the existing data base. A small working meeting was held in our laboratory at FCRF where 2D lymphocyte gels were compared by Dr. Lester and Dr. Leigh Anderson to test the feasibility of identifying the same spots in gels prepared in different laboratories. The consensus was that although there were substantial differences between gels, it was feasible to identify many proteins in gels between laboratories. On another visit Dr. Lester gave a seminar on his 2D gel work and used the new Xpix program on the SUN system to landmark lymphocyte leukemia gels as well as collaborate on future extensions to GELLAB-II.

Dr. Heinz Busse of the Univ. of Kiel, has been running GELLAB on his DEC10. He visited for a week where we collaborated on future extensions to GELLAB-II. Dr. Busse gave a seminar on his current work.

We are exploring with Dr. Michael Alley of NCI-PDR/FCRF the feasibility of using GELLAB-II at FCRF in part of his program.

We are exploring with Dr. Robert Paul of the Univ. of Hawaii Plant Physiology Dept. the feasibility of using GELLAB-II in his program to analyze 2D gels. We have had additional consultations with programmer staff and have sent them some of our gel analysis software.

Publications:

Lemkin PF. PSAIL: A Portable SAIL to C Compiler - Description and Tutorial. SIGPLAN Notices. 1988:1-23.

Lemkin PF. The GELLAB Papers - A Collection of Papers Describing the GELLAB-I System. NCI/FCRF, 1988:1-447.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08382-05 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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)*

Bruce Shapiro, Ph.D.,	Computer Specialist	IPS, LTB, NCI

Other Professional Personnel:

Lewis L. Lipkin, M.D.	Chief, Image Processing Section	LTB, NCI
Peter Lemkin, Ph.D.,	Computer Specialist	IPS, LTB, NCI
Morton Schultz	Senior Engineer	IPS, LTB, NCI
Jacob V. Maizel, Jr., Ph.D.	Chief, Laboratory of Mathematical Biology	NCI

COOPERATING UNITS *(if any)*

Dr. Ruth Nussinov, Institute of Molecular Medicine, Sackler Faculy of Medicine Tel Aviv University, Tel Aviv, Israel; Dr. Jacob Mazur, Program Resources, Inc., Frederick, MD.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Image Processing Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Work on nucleic acid structure has continued in a variety of collaborations and directions. Among these include the development of a fully integrated computer environment for the analysis of RNA secondary structure. The others have involved the development of new algorithms to help analyze these structures. One of the objects of this research is to make available an experimental computer workbench to allow a researcher to interactively pursue various areas of interest concerning RNA secondary structure in a coherent way. This includes both the use of facilities to explore in detail multiple structures as well as individual RNA structures. This system is forming the basis for an expert system which is permitting intelligent queries of relationships that exist in the RNA secondary structure problem domain involving various software/hardware complexes available at the Frederick Cancer Research Facility.

Several algorithms have been developed that involve RNA secondary structure analysis. These include several algorithms to measure structural similarity, an algorithm to determine how RNA structural energies are related to sequence composition and an algorithm to determine structural consensus.

Work has begun on a new algorithm for speeding up the prediction of RNA secondary structure by jumping over portions of structure that are not going to add very much to a given structure. Also, a new algorithm for secondary structure comparison based on precise tree matching is being developed.

The DNA structure analysis program which was enhanced to include rules on DNA melting was used to analyze procaryotic promoter regions. Using this system we were able to show that promoter regions appear to be related to the stabilizing energies of the promoter.

GPO 914-918

Project Description:

Major Findings:

A highly integrated system for the analysis of RNA secondary structure has been developed on a SYMBOLICS 3675 computer. One of the objects of this research is to make available an experimental computer workbench to allow a researcher to interactively pursue various areas of interest concerning RNA secondary structure in a coherent way. This includes both the use of facilities to explore in detail multiple structures as well as individual RNA structures. This system is forming the basis for an expert system which is permitting intelligent queries of relationships that exist in the RNA secondary structure problem domain involving various software/hardware complexes available at the Frederick Cancer Research Facility. The system currently has a large number of functions that permit the use of algorithms that reside on different nodes on the FCRF network. This includes the SUN'S, and VAXES and very shortly the CRAY XMP. These algorithms are invoked from the SYMBOLICS utilizing one common mouse and window system that reduces the users need to know the various software/hardware complexes. The user has the interactive capability to fold literally hundreds of structures and to cluster these structures to determine which are similar and which are not as well as which substructures are similar and which are not (see 1). The ability also exists to search for specific structural elements that are a function of global or semi-global structure, base pairing, local energies as well as sequence. The ability exists to graphically display structures that are generated. One may then interact with the display to get at various local structural elements. The structure may be labeled in different ways so that the important area of current interest may be viewed. Structures may be compared analytically, for example using a Boltzman distribution as well as visually. The system is in the process of being ported to Sun workstations.

New algorithms and methodologies have been developed that permit the clustering, searching for, and examining in detail of multiple RNA secondary structures from a structural as well as sequence standpoint. The object is to find structural similarities that could be correlated with functional sites. This includes the examination of how mutations affect structure and function in RNA. Examination of RNase III sites in several different genes as well as the regulation effects of L1 and L11 genes are being examined in detail using the system with its new set of algorithms (with Margalit, Maizel-LTB, Oppenheim-Hebrew University, Takiff-LMO). Mutations may be generated that preserve the amino acid coding sequence and then these structures are then compared to determine which ones are similar to the wild type or other alternate structures. These in turn suggest mutational experiments to perform in the laboratory to determine how the mutants perform in comparison with the predicted models. These types of collaborations are currently underway with Howard Takiff of LMO which involve RNase III sites in various genes including T7, T3 and Lambda and Amos Oppenheim at the Hebrew University in Israel involving RNase III sites specifically in the C3 gene of Lambda. Comparative studies are also underway with structures published in the

literature, e.g., L1 and L11. A preliminary examination of the L1/L11 data shows some good correlations to the published experimental results.

Work has been pursued involving computer experiments to determine how RNA conformational energies are related to sequence composition. An algorithm has been developed that can determine what the average energy would be for a given sequence composition. This is being utilized to determine the significance of a folded RNA molecule compared to what would normally be expected given its base composition (with Chen, Le, Currey and Maizel-LTB). This type of data is also be looked at to possibly speed up the RNA folding algorithm by jumping over insignificant portions of structure (with Nussinov, Le and Maizel).

Work has been pursued involving the determination of a RNA secondary structure consensus. An algorithm has been developed that allows one to determine the structurally significant conformations from a group of conformations thus forming a consensus (with Le, Owens, Nussinov, Maizel). The object of this algorithm is to determine what structures within a given sequence are most stable under the influences of folding and to extract those structures to form a consensus structure. The consensus may also be determined by looking at preserved structures generated from suboptimal foldings.

The DNA structure analysis program which was enhanced to include rules on DNA melting and energy calculations from Breslauer rules for helix to coil transitions were used to analyze procaryotic promoter regions (with Margalit, Jernigan, Nussinov, and Owens). Using this system we were able to show that promoter regions appear to be related to the stabilizing energies of the promoter. We found that the -10 promoter sites were less stable than other parts of the sequence. Also, we found supportive evidence in regard to experimental mutations within the -10 region and the direction of promoter activity (see 2).

Work has begun on utilizing a new tree comparison algorithm to do flexible and accurate comparisons of RNA secondary structures. This would include the ability to determine precisely where differences reside between to structures and to place a measure on these differences (with Zhang and Shasha-Courant Institute). The work has evolved out of a lecture I gave to a graduate course established at the Courant Institute at NYU with participation of various NIH and non-NIH lecturers. The algorithm mentioned here will be included in the above described system to get an even better handle on comparative structures.

Publications:

Shapiro BA. An algorithm for comparing multiple RNA secondary structures. CABIOS, in press, 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08384-02 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Mapping Chromosomal-Sized DNA

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

James L. Cornette, Ph.D. Expert LTB, NCI

COOPERATING UNITS *(if any)*

Charles DeLisi, Chairman, Department of Biomathematical Sciences, Mount Sinai Medical School, New York, New York 12028

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We examine the problem of reconstructing the order of DNA fragments that are produced by restriction digests as they appear in the original DNA sequence. Established procedures that work for DNA segments in the 5-10 Kb range do not work for human chromosomal size DNA (approx. 100,000 Kb). It is assumed that multiple copies of an original DNA segment have been digested with one or more enzyme digests in such a way that overlapping fragments of the original DNA are created. The size of the fragments should be appropriate to cloning in some bacterial system or in yeast (where larger fragments, 200Kb, have been cloned), and a "library" consisting of clones of these fragments is assumed. The overlapping fragments may be obtained by partial digestion of the DNA by a single enzyme or total digestion by two or more enzymes used individually. The critical step is to identify as many pairs of overlapping fragments as possible. If the library covers the chromosomal DNA and all intersecting pairs can be identified, a minimal sequence of overlapping fragments reaching from one end of the DNA to the other may be obtained. Determination that two fragments overlap can be made by further digestion of each of the fragments into smaller pieces and observing that two pieces, one from each fragment, are identical.

Questions that arise are what numbers and sizes of fragments are produced by different digestion protocols, what fraction of the DNA is covered by the clonable fragments, and what is effectiveness of different procedures for determining that pieces of fragments are identical.

GPO 914-918

Project Description

Major Findings

We have evaluated reference 1 a rigorous and complete algorithm for mapping chromosomal DNA based on first obtaining single digest fragments from complete digestion by each of two endonuclease digests, A and B. Subsequently, each single digest fragment is digested with the alternate digest, yielding duplicate sets of double digest fragments, (A,B) and (B,A). Each fragment in (A,B) is then identified with its duplicate in (B,A), each such pair indicating an overlap of an A-digest fragment with a B-digest fragment. An ordered sequence of overlapping single digest fragments may thus be identified that covers the chromosomal DNA. Limitations on the method are due to the limited size of DNA fragments that can be cloned and incorrect identification of (A,B) and (B,A) fragments. For human chromosomal size DNA, depending on the cloning technique and the lengths of the recognition sites of enzymes A and B, it may be that at most 60 percent of the DNA is covered by clonable fragments. Cloning in yeast may enable cloning of fragments in lengths up to 200 Kb, however, and using enzymes with recognition sites of length 7 could provide over 90 percent coverage of the DNA. Pieces of fragments can be identified by determining which members of a panel of probes bind to the pieces, and a panel of some 30 probes used in combination with estimates of lengths of the fragments may be sufficient to distinctly identify pieces. A more effective identification technique is to sequence the twenty nucleotides at the, say, 5' end of the pieces.

A similar algorithm for a partial digest protocol has been examined. The major difficulty here is that some 8 to 10 times as many fragments (approximately 100,000) may have to be cloned.

These algorithms have been evaluated for randomly generated DNA sequences, and next should be examined for sequences that incorporate real DNA characteristics, particularly inclusion of repetitive components.

Publications:

Cornette JL, DeLisi C. Some mathematical aspects of mapping DNA cosmids. Cell Biophysics 1988;12:271-293.

Cornette JL, DeLisi C. Mapping chromosomal-sized DNA. In: Proceedings of the 12th World Congress on Scientific Computation, Paris: International Association for Mathematics and Computers in Simulation, July, 1988, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08385-02 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

DNA Conformations in Control Regions

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

Robert L. Jernigan, Ph.D.,	Theoretical Physical Chemist	LTB, NCI
Hanah Margalit, Ph.D.,	Visiting Fellow	LTB, NCI
Bruce A. Shapiro, Ph.D.,	Computer Specialist	LTB, NCI
John Owens, Ph.D.,	Microbiologist	LTB, NCI
Ruth Nussinov, Ph.D.	Consultant	LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Extracting molecular details of the mechanism of promoter function in transcription is a subject of broad interest. We have chosen to look at one of the simplest aspects of this process, namely, the strength of strand pairing in the promoter region. Specifically, the stability of the DNA double helix was determined in the vicinity of the promoter by computing the free energy for strand separation as a function of dinucleotide free energy values taken from the calorimetric measurements of Breslauer et al. The stability of 168 E.coli promoter regions was studied within a window of +/- 250 nucleotides on either side of the transcription start sites. We found that for this set of promoters the -10 region was significantly the least stable. There is no correlation between the free energies and the rates of RNA polymerase-promoter open complex formation measured for 25 promoters. We also compare the free energies of 121 promoter mutations across the -35 and -10 consensus regions with the free energies of the corresponding wild type sequences. These pairwise mutant-wild type comparisons provide a particularly good test since the examined sequences differ only in one nucleotide so that all other sequence dependent effects are fixed. About 80% of the mutations in the -10 region that show increased/reduced promoter activity are less/more stable than the wild types. The observed high free energy peak, as well as the mutation data, strongly support the conjecture that the instability, or melting properties, of the -10 region play a significant role in promoter function.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Major Findings:

For a collection of 168 promoters we showed that the -10 region is the least stable, within a 500 nucleotide span around the transcription initiation site. We revised our database to include only promoter sequences for which the transcription start positions have been identified experimentally. The present database of 168 E.coli promoters is based on the recent compilation of promoter sequences (Harley and Reynolds, 1987) and on GENBANK release 52. The peak in frequency of TA (the least stable dinucleotide) in the -10 region might suggest that one of its roles is in facilitating the "melting in" step in the open complex formation.

In a data base of 121 up and down promoter mutations about 80% of the mutations in the -10 region that show increased/ reduced promoter activity are less/more stable than the wild types. There is no correlation between the type of mutation in the -35 region and the direction of free energy change. Also, the -35 region is not relatively unstable.

The mutation analysis points to a possible dependence of promoter activity on the -10 region helix stability. A possible mechanism that could cause such a dependence is the following:

1) The enzyme interacts with the -35 region to form the closed complex.
2) The -10 region is opened by thermal fluctuations.
3) The enzyme interacts with the appropriate strand in the -10 region to form the open complex.

A kinetic formulation for this process, shows a direct relationship between the free energy and the rate of open complex formation. However, We did not find a linear correlation between the free energies and the rates of open complex formation (available for 25 promoters), implying that the stability of the DNA is not the only factor that determines the rate of the process. The last step might be rate limiting and is probably sequence dependent as well.

The kinetic analysis compares activities of different genes and the variation among them is probably a result of various sequences affecting the different steps that lead to an open complex formation. In the mutation analysis the activities of a wild type and a mutant of the same gene are compared. The only difference between the two promoters is a single point mutation. All the sequence dependent factors affecting transcription rate are identical except the one at the position of the mutation. The strong correlation between the type of mutation and the direction of free energy change in the -10 region indicates that the ease of melting directly affects the transcription rate.

PUBLICATIONS:

Margalit H, Shapiro BA, Nussinov R, Owens J, Jernigan RL. Helix Stability in Prokaryotic Promoter Regions. Biochemistry, 1988 in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08386-02 LTB

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Primary Structure and Topology of Beta 1-4 Galactosyltransferase

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

Pradman K. Qasba, Ph.D. Research Chemist LTB, NCI

Other Professional Personnel:

Arni Masibay Intramural Research Training Fellow LTB, NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	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 on the primary structure of beta 1-4 galactosyltransferase (1-4 galtransferase) continued in this year. To deduce the complete amino acid sequence of 1-4 galtransferase, genomic clones were isolated. NH_2-terminal end sequence derived from the cDNA and genomic clones showed that the 1-4 galtransferase is synthesized as a larger precursor. The soluble and secreted form is missing approximately 72-residue-NH_2-terminal peptide which contains the membrane anchoring domain as well as the potential protease susceptible sequence - Arg-x-x-Arg-Arg-Leu-. The milk enzyme can be generated from the larger precursor by proteolytic cleavage between the NH_2-signal-anchor and the catalytic domain. The topology of the Golgi and cell surface 1-4 galtransferase can be predicted to be similar to a class of membrane proteins, like the transferrin receptor, the asialoglycoprotein receptor, the beta-galactoside alpha-2, 6-sialyltransferase, the influenza virus sialidase etc., which have an NH_2-terminal "stem" structure extending from the luminal side of the transmembrane bilayer to the cytoplasmic side. The bulk of the protein structure, which carries the main function, faces the lumen side.

Project Description:

Major Findings:

Amino-termina 72-residue peptide is missing from the soluble form of 1-4 galtransferase: Sequence analysis of the cDNA clones, longer at the 5' end than our previously reported clones, showed that 1-4 galtransferase has extra residues at the amino terminus of the protein than are present in the soluble and secreted form of the protein. A full-length cDNA clone which would encode the entire NH_2-terminal peptide sequence of the galtransferase, containing NH_2-terminal methionine, could not be isolated from our previously described Okayama-Berg bovine mammary gland cDNA library nor could we isolate such a clone from bovine mammary gland cDNA library prepared λ-Zap in our laboratory. Therefore, we isolated bovine galactosyltransferase genomic clones to analyze the remaining NH_2-terminal end sequence of 1-4 galtransferase. The results of these sequence analysis show that 1-4 galtransferase is synthesized as a 401 residue protein which contains an NH_2-terminal 72-residue-peptide which is missing from the secreted and soluble form of the enzyme. The remaining 320-residue protein, carries the catalytic domain and is the soluble form of the protein.

Topology of 1-4 galtransferase: The cDNA derived protein sequence shows that there is a hydrophobic domain at the NH_2-terminal end which might serve as a signal membrane anchorage sequence. Based on the current knowledge of the signal-peptide mediated translocation of the protein across the endoplasmic reticulum, the amino-terminal end would face the cytoplasm. The remaining 320-residue COOH-terminal portion of the protein which carriers the catalytic domain faces the lumen. Thus the topology of the Golgi and cell surface 1-4 galtransferase is similar to that of a certain class of membrane proteins, e.g. the transferrin receptor, the asialoglycoprotein receptor, the beta-galactoside alpha-2, 6-sialyltransferase, the influenza virus sialidase, etc., which have the bulk of the protein carrying the main function facing towards the lumen side while the NH_2-terminal signal-anchors extending as a "stem" structure from the luminal side of the transmembrane bilayer to the cytoplasmic side.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08387-01 LTB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Fracture-Flip

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:

Catarina Andersson Forsman, M.D., Ph.D.	Visiting Fellow	LTB, NCI
Kazushi Fujimoto, Ph.D.	Visiting Associate	LTB, NCI
Paulo Pimenta, Ph.D.	Visiting Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

Institute of General Pathology, Univ. of Rome School of Med., Rome, Italy (M.R. Torrisi, A. Pavan).

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, FCRF, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.8	2.8	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.)*

We introduce fracture-flip, a method to obtain high resolution views of membrane surfaces. The method, a corollary of label-fracture (Pinto da Silva & Kan, J Cell Biol. 99: 1156, 1984), is based on the stabilization of the exoplasmic halves of membranes by carbon evaporation. Inversion of these casts followed by Pt/C imaging leads to new views of the cell surface at macromolecular resolution. Because of the obvious promise of fracture-flip we redirected the activity of our section and concentrated most of our research effort in the development and application of the new method. As a result, we have now completed and sent for publication three studies that should be published before the end of 1988. The first describes Fracture-flip; the second reports the migration of surface macromolecules during the formation of coated pits in spreading macrophages; the third describes the surface of boar sperm. We have completed the experiments of other studies including: ultrastructure of the nuclear envelope; visualization of cytoplasmic surfaces of membranes comparative studies of Leishmania major from infective and non-infective phases; mast cell secretion; cell capping ultrastructure of nerve cells in culture; application of colloidal gold to fracture-flip. We are confident that fracture-flip will substitute high-resolution scanning electron microscopy and of cell surfaces will provide macromolecular resolution images of the structure and topochemistry of biological membranes.

GPO 914-918

Project Description:

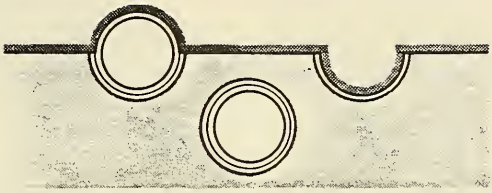
Major Findings:

Fracture-flip is a corollary of label-fracture. With label-fracture we showed that the split membrane halves remain attached to the replica. Thus, exoplasmic halves of a membrane can be labeled (pre- or post-fracture) and superimposed, coincident images of the label and of the Pt/C replica of the E face are observed. We reasoned: if the outer half of the membrane remains attached to the replica it should be possible to stabilize the fracture face with (electron translucent) carbon, thaw, wash and invert the replica (FLIP) to expose the actual surface of the membrane. Then, we could obtain its high resolution cast by Pt/C evaporation.

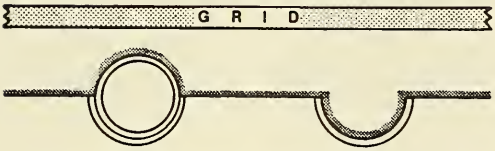
The sequence of steps is illustrated below. Fracture-flip is now giving us in a constant, rapid stream images of a new world. To interpret the replicas some adaptation is required. For instance: cells remain attached to the replica by their P faces or by cross fractured interfaces; transmembrane proteins that, on fracture, partition with the P half are not to be seen. Stereo pairs provide striking images of this new reality in a way that never happened with freeze-fracture. The cell surface is the surface of an object, intrinsically familiar as any; in contrast, the fracture face is but the signal of a process.

The routine resolution of Fracture-flip is about 2-3 nm, much higher than that of SEM of biological specimens. Fracture-flip allows also the observation of the surfaces of intracellular membranes (after isolation of organelles) and is easily combined with colloidal gold cytochemistry.

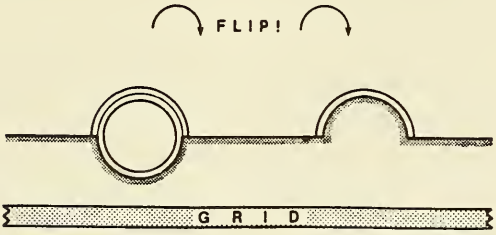
We used Fracture-flip to examine at macromolecular resolution the cell surfaces of the human erythrocytes, lymphocytes and thrombocytes. Human lymphocytes and thrombocytes were isolated using Ficoll-Paque (Pharmacia), fixed in 1.5% glutaraldehyde in PBS, rinsed in the same buffer and glycerinated. The specimens were fracture-flipped. At low magnification the surface of erythrocytes appears smooth. Closer examination of the surface of erythrocytes reveals a high density of very small (3-5 nm) granularities which contrasts the smoother adjoining crossfractured glycerol. By Fracture-flip human lymphocytes appeared as dome shaped structures, with many cross fractured cell accidents (e.g. microvilli). These villi dry, contract, shrivel and the plasma membrane detaches and reveals dried cytoplasm. The surface of human lymphocytes is covered by 10-17 nm particles distributed over the cell body and the microvilli at a density of approximately 1500/μm . The surface of thrombocytes displays a flocculent texture, with loose aggregates (about 25-30 nm diameter) of smaller structures, at a density of approximately 2500/μm . Fracture-flip easily shows the indentations and openings of the open canalicular system (OCS).



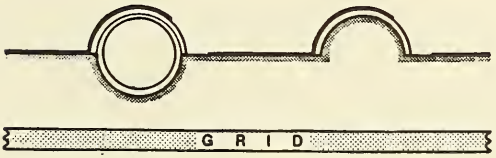
Cells are fractured and carbon cast



Specimens are thawed, washed and picked on grids



Membrane surfaces are now on top



Pt/C images cell surfaces

We show that carbon casting of freeze-fractured cells prevents deformation of cell shape. Pt/C replication (unidirectional or rotary) of the cell surface easily reveals new, high resolution, images of the cell surface. We combined fracture-flip with immunocyto-chemistry to show the distribution of IgG Fc-receptors as labeled by colloidal gold. The combination of Fracture-flip with colloidal gold cytochemistry will permit the localization of receptors and antigens cytochemically defined structures to morphologically defined structures.

We studied the macromolecular dynamics of the cell surface of rat alveolar macrophages during spreading on a substrate, a process that involves the formation of numerous coated pits. We used "Fracture-Flip" to prepare high resolution platinum shadowed replicas of membrane surfaces. Our observations show the following sequence of events associated with coated pit formation: at 4 C the cell surface of macrophages is covered with a moderate density of particulate components, with most ranging from 10 to 25 nm in diameter. These particles appear to be randomly distributed over the cell surface. Incubation of adherent cells at 37°$C_2$for 15 minutes results in the formation of large loose clusters (area 0.5-4 μm) of particles on the adherent surfaces. After incubation of macrophages for 30 minutes at 37°C, these clusters become tighter and, eventually form circular depressions (200 nm to 300 nm in diameter) which we interpret as a sequence of invagination. After 60 minutes, the depressions become much steeper. At this time surface particles can be observed on the intervening non-invaginated regions, and the peripheral region of the adherent membrane, as well as the free membrane. Fracture-flip reveals the presence of structures undetected in previous electron microscopic studies and provides ultrastructural evidence for the clustering of surface macromolecules that is involved in the formation of coated pits.

We used Fracture-flip to produce new, macromolecular resolution images of the surface of boar spermatozoa. Over the head, acrosomal and postacrosomal regions display sharply demarcated subtly different surface textures. The rim is particle poor as is a region above the oblique cords over the posterior ring. The tail shows two morphological distinct domains: 1) the principal piece is covered by a high density of parallel-helical strands and a high density of large globular particles; 2) the midpiece and the neck are covered by smaller particles with apparent random distribution. Rectangular surface specializations frequently seen near the annulus display waffle-like texture. With the notable exception of the parallel-helical strands of the principal piece the fracture-flip images of the boar spermatozoa can be related with the freeze-fracture morphology of its plasma membrane.

Publications:

Andersson-Forsman C, and Pinto da Silva P. Fracture-flip: New, high resolution images of cell surfaces after carbon stabilization of freeze-fractured membranes. J Cell Sci, In press.

Andersson-Forsman C, and Pinto da Silva P. Fracture-flip views the surface of sperm cells macromolecular resolution. Proceedings of the Ninth European Congress of Electron Microsc. York, England: European Congress of Electron Microscopy, 1988, In press.

Andersson-Forsman C, and Pinto da Silva P. The surface of human blood cells as revealed by fracture-flip. Proceedings of the Ninth European Congress of Electron Microsc. York, England: European Congress of Electron Microscopy, 1988, In press.

Pinto da Silva P, Andersson-Forsman C, and Fujimoto K. Fracture-Flip: Where freeze-fracture is turned upside down and comes from behind the looking glass to reveal the cell surface. Proceedings of the Ninth European Congress of Electron Microsc. York, England: European Congress of Electron Microscopy, 1988, In press.

Andersson-Forsman C, and Pinto da Silva P. High [illegible] surface views of spermatozoa as revealed by fracture-flip. J Cell [illegible] press.

Fujimoto K, and Pinto da Silva P. Macromolecular dynamics of the cell surface during the formation of coated pits is revealed by fracture-flip. J Cell Sci, In Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08388-01 LTB

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 charecters or less. Title must fit an one line between the borders.)*: Expression of Beta 1-4 Galtransferase

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

Pradman K. Qasba, Ph.D. Research Chemist LTB, NCI

Other Professional Personnel:

Arni Masibay Visiting Fellow LTB, NCI

COOPERATING UNITS *(if any)*

Dr. T. Sreevalsan, Department of Microbiology, School of Medicine, Georgetown University, Washington, D.C. 20007

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors B

☐ (a2) Interviews

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

Hormonal requirement and tissue specific expression of several molecular forms of beta-1-4 galtransferase is being investigated. In mammary gland its expression is modulated by lactogenic hormones and is also dependent on the functional differentiation of the mammary tissue.

The primary goal of this project is to identify cell lines which: a) do not produce 1-4 galtransferase, b) produce very low levels, and c) very high levels of the enzyme. Such cell lines will be used for transfection experiments to determine the structure-function relationship of galtransferase and alpha-lactalbumin, and also the hormonal regulation of expression of the enzyme.

In the present study we are: A) screening several cell lines for 1) the activity of 1-4 galtransferase, 2) the molecular form of the protein produced, 3) mRNA levels for the enzyme, and 4) mRNA size class produced; B) investigating the effect of steroidal and mitogenic hormones and their combinations on any of the parameters stated in A); C) determining cell-cycle dependent expression of the enzyme in Swiss mouse 3T3 cells.

Project Description:

Major Findings:

1-4 galtransferase expression in the mammary gland: mRNA and protein levels of the enzyme in rat increase during the late stages of gestation (18-19th day of gestation) and then continue to increase during lactation till day 12th of lactation. This increase parallels the production of alpha-lactalbumin, the protein which interacts with the enzyme to form lactose synthetase complex. On the other hand the c-Has-ras mRNA starts decreasing from the 19th day of gestation and continues to decrease during lactation.

Cell-cycle dependent expression of 1-4 galtransferase in mouse 3T3 cells: Expression of 1-4 galtransferase during different stages of cell-cycle in mouse 3T3 cells is studied in conjunction with the expression of other mRNAs during the cell's transit through G_1. 1-4 galtransferase is expressed during early G_1 period of these cells (2.5 hrs. after serum stimulation of the confluent cells). These mRNA levels then decline by the 6th to 8th hour of stimulation. Other mRNA species stimulated during this period have been identified. One of them being gamma actin-specific mRNA.

Expression of 1-4 galtransferase in established cell-lines: Expression of galtransferase is being studied in various tissues and cells lines. In the present study we are screening several cells lines for 1) the activity of 1-4 galtransferase, 2) the molecular form of the protein produced, 3) mRNA levels for the enzyme and 4) mRNA size class produced.

Publications:

Nieto A, Qasba PK, Nakhasi HL, Dhar R. Differential regulation of c-Ha-ras and c-Ki-ras gene expression in rat mammary gland. Carcinogenesis 1987 Dec., 8(12):1955-1958.

Masibay AS, Qasba PK, Sengupta DN, Damewood GP, Sreevalsan T. Cell-cycle-specific and serum-dependent expression of Gamma-actin mRNA in Swiss mouse 3T3 cells. Molecular and Cellular Biology 1988;8(6):2288-2294.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08389-01 LTB

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Structure-Function Relationship of Beta 1-4 Galactosyltransferase

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

Pradman K. Qasba, Ph.D.	Research Chemist	LTB, NCI

Other Professional Personnel:

Arni Masibay	Intramural Research Training Fellow	LTB, NCI
Anil Puri	Visiting Fellow	LTB, NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors B

☐ (a2) Interviews

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

Studies on the structure-function relationship of proteins, specifically of galactosyltransferases and their modifiers like alpha-lactalbumin, have been initiated. For such studies, we are producing the normal and altered proteins by the recombinant DNA methodology. A T7-promoter and a polylinker DNA sequence was inserted into a baculovirus vector adjacent to the polyhydrin promoter. Such constructs are used for the production of normal and altered proteins in, a) baculovirus infected insect cells, or b) frog oocytes by injecting the cDNA specific mRNA transcribed in vitro by T7-polymerase. We have also engineered the cDNA sequences of galactosyltransferase and alpha-lactalbumin, in such a way that the expression of the cDNA sequence have a potential to produce either soluble and secreted forms, or membrane anchored forms of the protein.

Project Description:

Major Findings:

To determine a "functional" region or a unit of a protein, we are, based on recombinant DNA technology, developing a general method to produce the normal and altered proteins. Baculovirus vector system is engineered in such a way that the same vector can be used to produce protein in quantities enough to test its function, and as well to produce the engineered protein in large quantities for determining various physical properties and X-ray crystallo-graphic studies.

Construction of a modified Baculovirus Vector: We synthesized on the Gene-Assembler a 62-mer DNA sequence which contains T7-promoter and a polylinker sequence. This was inserted into a baculovirus plasmid vector, pVL941 (provided by Dr. Max Summers), into the BamHl site, adjacent to the polyhydrin promoter. In these modified plasmid vectors, the cDNA sequences are inserted into the polylinker site, 3' to the polyhydrin and T7 promoters. Expression of any cDNA sequence, inserted into the polylinker site, is then either directly under the control of baculoviral polyhydrin promoter or T7-promoter. These constructs are now being used as follows in our laboratory: a) for the in vitro synthesis of mRNA corresponding to the inserted cDNA with T7-polymerase and the synthesized RNA is injected into the frog oocytes to synthesize proteins for testing the enzymatic function of the normal or altered protein and for the substrate binding specificities. b) for the production of normal or altered proteins in large quantities in the insect cell monolayer which are cotransfected with the wild type baculovirus. The expression of the cDNA in the infected insect cells is directed by the baculoviral polyhydrin promoter present 5' to the cDNA sequence.

Engineering of the cDNA sequences of 1-4 galtransferase and alpha-lactalbumin: We have engineered the 1-4 galtransferase cDNA sequence in such a way that some of the sequences have a potential to produce a 320-residue long soluble and secretory form of the enzyme, and others a soluble form of enzyme with alpha-lactalbumin signal peptide sequence and others a 401-residue long membrane anchored form of the protein.

SUMMARY STATEMENT

LABORATORY OF MOLECULAR BIOLOGY

DCBD, NCI

OCTOBER 1, 1987 to SEPTEMBER 30, 1988

The Laboratory of Molecular Biology uses genetic and molecular biological approaches to study gene activity and cell behavior and to develop new approaches to the diagnosis and treatment of human disease.

Genetic Analysis of the Multidrug Resistance Phenotype in Tumor Cells

Resistance to multiple drugs is a major impediment to the successful chemotherapy of human cancers. To investigate the genetic and biochemical basis for this multidrug resistance (MDR) phenotype, Dr. M. M. Gottesman and Dr. I. Pastan developed a model system using the cultured KB cell, a human carcinoma cell line selected independently for resistance to high levels of either colchicine, adriamycin or vinblastine which is cross-resistant to colchicine, adriamycin, vincristine, vinblastine, puromycin, and actinomycin D. This resistance results from expression of the MDR1 gene which encodes a 170,000 dalton membrane glycoprotein (P-glycoprotein) which is a multidrug transport protein. ATP-dependent transport of vinblastine has been demonstrated using membrane vesicles from MDR cells. Expression vectors into which the MDR1 cDNA are cloned confer the complete MDR phenotype on drug-sensitive cells, as does a retrovirus carrying the MDR1 gene. Expression of MDR1 RNA and P-glycoprotein occurs in normal kidney, liver, colon, and adrenal and in tumors derived from these tissues which are intrinsically resistant to chemotherapy as well as several other tumors. Acquired drug-resistance in childhood leukemia, neuroblastoma, rhabdomyosarcoma and in pheochromocytoma may be associated with increased MDR1 RNA levels. New strategies are being devised to overcome resistance due to expression of the MDR1 gene.

Regulation of Activity of the EGF Receptor Gene

The EGF receptor (EGFR) is overexpressed in many human tumors implicating it in the pathogenesis of some cancers. To test the role of the EGFR in cancer, Dr. I. Pastan and G. Merlino constructed a retrovirus encoding the EGFR which was shown to cause EGF dependent transformation of 3T3 cells. To study regulation of the EGFR gene, the promoter region was analyzed and shown to contain many transcription factor binding sites including six Sp1 sites and sites for three other factors. Factor ETF1 stimulates EGFR transcription, has a M_r of 120 kilodaltons, and binds to region -230 to -250. Factor ETF2 stimulates both EGFR and SV40 transcription and binds to -120 to -145. EGF has been shown to raise EGFR RNA levels in KB cells. This effect appears to be due to an increase in EGFR RNA half life. Understanding how expression of the EGFR is regulated and the role of the EGFR in cancer has led to new strategies of cancer treatment.

Immunotoxin and Oncotoxin Therapy of Cancer Cells

Dr. I. Pastan, Dr. M. C. Willingham and Dr. D. FitzGerald have studied how Pseudomonas exotoxin (PE) or genetically modified forms of PE have been attached to monoclonal antibodies (mAbs) or growth factors to create cell-specific cytotoxic agents. Native PE attached to mAb OVB3 is cytotoxic to ovarian cancer cells, and to ovarian cancer in mice, and is being currently tested in women with ovarian cancer. Because overexpression of the EGFR can cause tumor formation and is observed in many cancers, mutant PE molecules in which domain I has been replaced by TGFα have been created by gene fusion and the chimeric protein, TGFα-PE40 produced in E. coli. TGFα-PE40 is very cytotoxic to cells with EGF receptors. Similarly, because the IL2 receptor is overexpresses in adult T cell leukemias, IL2-PE40 was created. IL2-PE40 is cytotoxic to cells with IL2 receptors. Such molecules may be useful in the elimination of tumor cells or other cells responsible for several human diseases.

Development and Uses of Eukaryotic Vectors

Dr. Bruce Howard studies the growth regulation in mammalian cells. The investigation of growth inhibitory activity detected by DNA-mediated gene transfer is emphasized within this framework. Two techniques, magnetic affinity cell sorting (MACS) and fluorescence activated cell sorting (FACS) have been used in conjunction with gene transfer to measure transient growth regulation mediated of exogenous DNA sequences. Several results of interest have been obtained: i) at the polypeptide level, development of an efficient transfection protocol for human embryo fibroblasts has led to initiation of a project in which cDNA libraries are screened for genes involved in senescence and tumor suppression, ii) at the RNA level, evidence has been obtained for growth regulation, both positive and negative, by RNA polymerase III-encoded transcripts; mutational studies to derive structure/function relationships for 7SL RNA- and Alu-mediated growth control are in progress, iii) at the DNA level, cytotoxicity has been found to follow introduction of retroviral, e.g., HIV, long terminal repeat sequences; this finding may have implications for mechanisms of viral pathogenicity.

Structures and Roles of Transformation-Sensitive Cell Surface Glycoprotein

Dr. K. M. Yamada is studying fibronectin, a major cell surface and extracellular matrix glycoprotein involved in cell adhesion and migration. A synthetic peptide containing the key fibronectin adhesive recognition sequence Gly-Arg-Gly-Asp-Ser administered intravenously substantially enhanced survival of mice challenged concurrently with melanoma cells in an experimental metastasis assay. Since renal clearance of the peptide was very rapid, conjugation of a peptide derivative to a carrier is being tested as a means of increasing its time in the circulation. In addition, fibronectin was shown to contain a second binding site that synergizes with the original peptide to produce full adhesive affinity and efficient transmembrane effects of the peptide on cytoskeletal organization. Mutants produced by site-directed mutagenesis or deletion of each site displayed >96% losses of activity, and the two types of mutants synergized in vitro for adhesion and promoting organization of actin microfilament bundles. Studies of this region may yield a novel class of inhibitors of fibronectin function. Two

other cell adhesion sequences are present in certain fibronectin molecules due to alternative mRNA splicing. Besides melanoma cells, embryonic cells from the peripheral nervous system were found to use this cell-type specific domain, which may be recognized only by cells derived originally from the neural crest. The Gly-Arg-Gly-Asp-Ser peptide and related analogues inhibited adhesion of embryonic cells involved in somite and heart formation.

Functions, Structure, and Regulation of Receptors for Cell Adhesion Proteins

Dr. K. M. Yamada is studying how cells interact with extracellular matrix molecules by specific receptors. Membrane binding molecules for fibronectin and collagen have been characterized. The beta subunit was synthesized as a smaller precursor that required a lengthy maturation period involving asparagine-linked oligosaccharides. Maturation resulted in an activation of fibronectin-binding function and secretion onto the cell surface. Levels of both alpha and beta subunits of the human fibronectin receptor were regulated by transforming growth factor β, which induced synthesis 20- to 40-fold. Fibronectin receptors on the cell surface were shown to exist in a transmembrane orientation. They are often diffuse on rapidly migrating embryonic cells and avian or human tumor cells, but clustered at adhesion sites of stationary cells. Roles for the fibronectin receptor were shown in embryonic amphibian gastrulation and human platelet function. A protein that was decreased after malignant transformation was shown to be globular monomer enriched in basic amino acids, with a unique amino-terminal 36-residue sequence containing a novel 7-mer tandem repeat unrelated to other heat shock proteins. Heat shock induces levels of mRNA. Two putative sequential biosynthetic precursors of this hsp47 protein were identified, both of which were fully biologically active in binding to a collagenous ligand.

Synthesis and Function of a Transformation-Dependent Secreted Lysosomal Protease

Dr. M. M. Gottesman is studying cultured mouse fibroblasts which are malignantly transformed or treated with TPA or growth factors which synthesize and secrete the 39,000 Mr precursor to cathepsin L in large amounts. This purified procathepsin L, originally called MEP for major excreted 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 with a lysosomal localization. Overproduction of cloned cathepsin L in a nontransformed cell results in secretion of this lysosomal enzyme. Secreted procathepsin L can bind to the mannose 6-phosphate receptor of many cells and be endocytosed and processed intracellularly. In antigen presenting cells, procathepsin L uptake reduces the efficiency of antigen presentation, thereby interfering with immune response. Transformation, TPA and PDGF stimulate procathepsin L synthesis by increasing levels of procathepsin L specific mRNA transcription. A functional procathepsin L mouse gene was cloned and the promoter identified. A human procathepsin L cDNA has also been cloned and sequenced. Human procathepsin L mRNA has been detected in all normal human tissues analyzed.

Genetic and Biochemical Analysis of Cell Behavior

The mechanism of cAMP action on CHO cells has been studied by isolating CHO mutants resistant to growth inhibition by cAMP. Dr. M. M. Gottesman is conducting these studies. These mutants have defective cAMP dependent protein kinases with either altered regulatory (RI) or catalytic (C) subunits. DNA from cells carrying dominant cAMP-resistant defects can be used to transfer the cAMP-resistance phenotype to sensitive cells. Cosmid clones carrying the CHO RI subunit from cAMP resistant cells have been isolated. One of these clones has the coding sequence for the CHO RI subunit.

Morphologic Mechanisms of Organelle Function and Transformation in Cultured Cells

Dr. M. C. Willingham is studying how neoplastic transformation produces many changes in cell physiology, some of which have been studied using morphologic techniques. Light microscopic cytochemical methods were used to isolate and characterize monoclonal antibodies to antigens present on ovarian cancer cells for use in diagnosis and in the construction of immunotoxins for therapy of human cancer. Technical improvements have streamlined the morphologic hybridoma screening processing time to less than two days, allowing primary screening on cultured cells and secondary screening on tissue samples with the same initial 100 µl supernatant sample. P170, the product of the human MDR1 gene, was localized using antibody MRK16. In addition to its location in GI tract, adrenal, kidney, and liver, it was found in capillaries in the central nervous system. The reactivity of monoclonal antibody C219 that reacts with an epitope of P170 present on the inner surface of the plasma membrane was studied. The antibody cross-reacts with other proteins in some tissues, such as the heavy chain of cardiac and slow-twitch muscle myosin, in human, monkey, and rat tissues. Expression of human P170 introduced into MDCK cells was studied and found to be expressed in a polarized fashion on the apical surface of these cells, similar to its distribution in normal kidney proximal tubules.

Mechanisms of the Transport of Thyroid Hormones into Animal Cells

Dr. S.-y. Cheng is studying the following subjects: 1. High level expression of the endoplasmic reticulum-associate thyroid hormone binding protein (p55). A full-length p55 cDNA was inserted into a Harvey murine sarcoma virus-derived vector (pHTBr) and transfected into NIH 3T3 cells. Nine stable cell lines expressing p55 were identified. These stable cell lines could be used as a tool to study further the function of p55. 2. Characterization of a cytosolic thyroid hormone binding protein (p58). A cellular hormone binding protein (p58) was purified to homogeneity. Two hybridomas secreting monoclonal antibodies to p58 were isolated. Using these antibodies, p58 was found to be located in the non-membranous cytoplasm (cytosol). Its role in the intracellular transport of T_3 is being investigated. 3. Antipeptide antibodies to the T_3 nuclear receptor. Recent studies have shown that c-erbA protein is the T_3 nuclear receptor. To study its tissue distribution and subcellular localization, antipeptide antibodies to the human placenta c-erbA (hc-erbA-β) protein were prepared. Two antibodies specifically reacted with the hc-erbA-β protein synthesized in vitro and immunoabsorbed the T_3 binding activity of the T_3 nuclear receptors isolated from placenta tissue and cultured cells.

The Transgenic Mouse as a Model System to Study Gene Function and Regulation

Dr. G. T. Merlino and Dr. I. Pastan have developed lines of transgenic mice to study two important aspects of cancer: the ability of malignant cells to escape destruction by chemotherapy, and the role of proto-oncogenes in the initiation of the transformed phenotype. A gene (MDR1) encoding a multidrug transporter (P-170) capable of conferring multidrug resistance was microinjected into one-cell mouse embryos and germ line transgenic mice established. Expression of the MDR1 gene was detected in the normally drug-sensitive bone marrow. Studies are underway to determine if transgenic mice overexpressing the MDR1 gene in bone marrow are more resistant to chemotherapy. The epidermal growth factor (EGF) receptor is the cellular homolog of the avian erythroblastosis virus erbB oncogene product. To elucidate the role that the EGF receptor plays in normal and transformed cells, transgenic mice were generated that carry germ line-integrated EGF receptor genes. These mice are being analyzed for EGF receptor gene expression, and scrutinized for the appearance of cancer.

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

Work on this project has been temporarily halted.

Regulation of the gal Operon of Escherichia coli

Dr. S. Adhya is studying the mechanism by which the Gal repressor protein negatively regulates the expression of the gal operon of E. coli. Repression requires binding of Gal repressor to two operator sites, O_E and O_I, which are separated from each other by 114 bp and flank two promoters of the operon. Repressor binding to O_E and O_I is co-operative. However, occupation of the operators by repressor is not sufficient to achieve normal repression and requires an interaction between the two operator-bound repressor molecules. Replacement of one of the gal operators by a lac operator causes derepression of the gal operon in the presence of both Gal and Lac repressors, whereas, substitution of both operators by lac operator sequences re-establishes repression. It has been proposed that the two repressor molecules bound to the spatially separated operators associate giving rise to a higher order structure containing a DNA loop with bound repressor, CRP and RNA polymerase. The DNA in this complex is altered and inactive for transcription. The following results are consistent with this model. 1. By analyzing repressor mutants which bind to DNA but do not associate and, thereby, fail to bring about repression, it has been demonstrated that repressor-repressor contact is needed for repression. 2. It was shown that repressor binding induces structural changes in DNA including DNA bending and change in circular dichrosim spectra.

Genetic Regulatory Mechanisms in Escherichia coli and Its Bacteriophage

Dr. S. Adhya and Dr. S. Garges are examining how cyclic AMP binds to its receptor protein CRP and induces a conformational change (allosteric transition) in the protein and how the cyclic AMP·CRP complex modulates gene transcription. Cyclic AMP·CRP complex, and not free CRP, binds at or near many promoters of E. coli in a sequence-specific way and either represses or activates transcription. Several classes of mutations in the crp gene were

studied which identify the amino acids participating in the cyclic AMP-induced allosteric shift in the protein. On the basis of these results and the available three-dimensional structure of the protein, it was proposed that cyclic AMP brings about: (1) a hinge reorientation to eject the DNA-binding F α-helices; (2) proper alignment between two subunits; (3) an adjustment between the position of the two domains. Extensive amino acid substitution at some of these positions further supports the idea that hinge movement involving interhelical polar-polar interactions and/or Van der Walls repulsion are an essential part of the activation of CRP. It was further shown, by site-directed mutagenesis, that hydrogen bond formation between amino acids 171 and 63 participates in domain-domain alignment for CRP activation. Using a DNA bending vector developed in this laboratory, it was found that CRP binding induces DNA bending at all test promoters with a CRP binding site whether the promoters are activable or repressible by CRP. The following two findings strongly support the idea that CRP activates RNA polymerase by a direct protein-protein contact and not by sending a signal through DNA: (1) Substantially increasing the distance between the RNA polymerase binding site and the CRP binding site in lacP does not affect CRP activation of lacP. (2) CRP and RNA polymerase bind to DNA co-operatively.

Bacterial Functions Involved in Cell Growth Control

The role that protein degradation plays in control of cell growth was studied by Dr. S. Gottesman using mutants defective in ATP-dependent and independent protein turnover. E. coli lon mutants are defective in cell division regulation after DNA damage, and overproduce capsular polysaccharide. It was shown that an unstable positive regulator of capsule synthesis, RcsA, is stabilized in lon mutants. The RcsA function seems to be common to a number of gram-negative prokaryotes, suggesting a common mechanism for regulating different capsules in these strains. RcsC, a negative regulator of capsule synthesis, is a membrane protein and thus may act as a sensor for environmental signals controlling capsule synthesis. The gene for the ATP-binding subunit of a new two component ATP-dependent protease was isolated and chromosomal mutations in the gene, called clpA, generated. While cells devoid of clpA are healthy, the Clp protease seems to act as a back-up to lon in degrading abnormal proteins. A third protease, Alp, function has been identified, and the gene responsible has been mapped to 56 minutes on the E. coli chromosome.

DNA Replication in vitro

To study properties of origin specific initiation of replication in vitro, an enzyme system was developed by Dr. S. Wickner that replicates exogenously added plasmid DNA containing the initiation origin of bacteriophage P1. The interaction of RepA and DnaA proteins with the P1 origin DNA is being studied. Deletion analysis was used to determine the minimal DNA site required for initiation of replication. Since P1 normally exists stably as a unit copy plasmid, this system is being used to study the molecular mechanisms involved in the regulation of a stringently controlled replicon. In collaboration with D. Chattoraj (LB, NCI, NIH), it was shown that the RepA protein binds to plasmid control locus incA and simultaneously to the repA promoter in the origin region, causing the intervening DNA to loop. DNA looping very likely could provide the mechanism by which RepA bound to incA might exert repression.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08000-18 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

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:	I. Pastan	Chief, Laboratory of Molecular Biology	NCI
	G. Merlino	Senior Staff Fellow	LMB, NCI
Other:	M.C. Willingham	Chief, UCS	LMB, NCI
	A. Johnson	Staff Fellow	LMB, NCI
	Y. Jinno	Visiting Fellow	LMB, NCI
	R. Kageyama	Visiting Fellow	LMB, NCI

COOPERATING UNITS *(if any)*

D. Lowy	Chief, Laboratory of Cellular Oncology	DCBD, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	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 EGF receptor (EGFR) is overexpressed in many human tumors implicating it in the pathogenesis of some cancers. To test the role of the EGFR in cancer, a retrovirus encoding the EGFR was constructed and shown to cause EGF dependent transformation of 3T3 cells. To study regulation of the EGFR gene, the promoter region was shown to contain many transcription factor binding sites including six Sp1 sites and sites for three other factors. Factor ETF1 stimulates EGFR transcription, has a $M\bar{r}$ of 120 kilodaltons, and binds to region -230 to -250. Factor ETF2 stimulates both EGFR and SV40 transcription and binds to -120 to -145. EGF has been shown to raise EGFR RNA levels in KB cells. This effect appears to be due to an increase in EGFR RNA half life.

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

Major Findings:

Regions of the EGF receptor promoter important for transcriptional activity were identified by deletion mapping and a 465 bp region (-481 to -16) was found to support transcription in transfection studies with promoter CAT fusions. This 465 bp region of the EGF receptor gene promoter was characterized for nuclear protein binding by exonuclease III protection, gel retardation and DNase I footprinting. Eight nuclear protein binding sites were identified. Four of these sites were shown to bind the transcription factor, Sp1. The other sites may be occupied by novel factors.

To investigate the mechanism by which the EGFR is regulated, we developed an in vitro transcription system for the human EGFR oncogene using nuclear extracts of A431 human epidermoid carcinoma cells which overproduce the EGFR. The nuclear extracts were fractionated by several column chromatography techniques. We found that a DEAE-Sepaharose fraction contained a novel factor, termed ETF1 (EGF Receptor Transcription Factor), which bound to region -230 to -250 and specifically stimulated EGFR transcription five- to ten-fold. We have purified ETF1 using sequence-specific oligonucleotide affinity chromatography and shown by renaturing the protein eluted from an SDS-polyacrylamide gel that ETF1 is a protein with a molecular weight of 120 kDa. The purified factor specifically stimulated the transcription from the EFGR promoter in a reconstituted transcription system but had little or no effect on the SV40 promoter. These results suggest that ETF1 may play an important role in the regulation of the EGFR oncogene.

Another factor (ETF2) that binds to region -120 to -145 was partially purified by using heparin agarose and oligonucleotide affinity chromatography. Addition of this partially purified protein to a reconstituted transcription system in vitro increased EGF receptor expression three- to five-fold and SV40 expression two- to three-fold. This factor is currently being further characterized.

A third factor is being studied by G. Merlino (see Annual Report Number Z01 CB 08756-01 LMB).

We examined the expression of EGF receptor mRNA in regenerating rat liver. The EGF receptor mRNA was shown to decrease in the first 12 hours after partial hepatectomy but was increased considerably at 24 hours and 72 hours.

We previously reported that EGF increases EGF receptor mRNA levels in KB cells and the effect appeared to be at a post-transcriptional step. We have now measured the half-life on EGFR mRNA and shown that in cells treated with EGF, the half-life increased from 2-4 hours to |18 hours.

In collaboration with D. Lowy, we have constructed a retrovirus that carries and expresses a human EGF receptor cDNA. 3T3 cells infected with this virus contain large amounts of human receptor, undergo EGF dependent transformation in tissue culture, and form large tumors in nude mice indicating the EGF receptor can act as an oncogene when expressed at high levels.

Publications:

Kitagawa S, Obata T, Hasumura S, Pastan I, Cheng S-y. A cellular 3,3'5-triiodo-L-thyronine binding protein from a human carcinoma cell line: Purification and characterization, J Biol Chem 1987;262:3903-8.

Velu TJ, Beguinot L, Vass WC, Willingham MC, Merlino GT, Pastan I, Lowy DR. EGF-dependent transformation by a human EGF receptor proto-oncogene, Science 1987;238:1408-11.

Hwang J, Richert N, Pastan I, Gottesman MM. Mutant KB cells with decreased EGF receptor expression: Biochemical characterization, J Cell Physiol 1987;133: 127-34.

Popescu NC, Cheng S-y, Pastan I. Chromosomal localization of the gene for a human thyroid binding protein, Amer J Human Genetics 1988;42:560-4.

Johnson AC, Ishii S, Jinno Y, Pastan I, Merlino GT. EGF receptor gene promoter: Deletion analysis and identification of nuclear protein binding sites, J Biol Chem 1988;263:5693-9.

Johnson AC, Garfield SH, Merlino GT, Pastan I. Expression of epidermal growth factor receptor proto-oncogene mRNA in regenerating rat liver, Biochem Biophys Res Commun 1988;150:412-8.

Kageyama R, Merlino GT, Pastan I. Epidermal growth factor (EGF) receptor gene transcription: Requirement for Sp1 and an EGF receptor-specific factor, J Biol Chem 1988;263:6329-36.

Jinno Y, Merlino GT, Pastan I. A novel effect of EGF on mRNA stability. Nucl Acids Res 1988; in press.

Kageyama R, Merlino GT, Pastan I. A transcription factor active on the epidermal growth factor receptor gene, Proc Natl Acad Sci USA 1988; in press.

Amano F, Pastan I, Gottesman MM. Genetic characterization of human KB cell lines resistant to epidermal growth factor - Pseudomonas exotoxin conjugates, J Cell Physiol 1988; in press.

Amano F, Gottesman MM, Pastan I. Epidermal growth factor-dependent growth of human KB cells in a defined medium and altered growth facotr requirements of KB mutants resistant to EGF - altered growth factor requirements of KB mutants resistant to EGF - Pseudomonas exotoxin conjugates, J Cell Physiol 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08001-17 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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 Regulation of Cell Behavior

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

PI: I. Pastan Chief, Laboratory of Molecular Biology NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Work on this project has been temporarily halted.

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08010-15 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 charecters 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 LMB, NCI

COOPERATING UNITS *(if any)*

Molecular Mechanism of Tumor Promotion Section, LCCTP, DCE, NCI
Cellular Regulation and Transformation Section, LTVB, DCE, NCI
Laboratory of Biochemistry and Metabolism, DIR, NIDDK

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Ultrastructual Cytochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	0.0	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, some of which can be studied using morphologic techniques. We have used morphologic methods to study three general areas: 1) We have used light microscopic cytochemical methods to isolate and characterize monoclonal antibodies to antigens present on ovarian cancer cells for use in diagnosis and in the construction of immunotoxins for therapy of human cancer. We had previously isolated OVB3, an antibody currently being evaluated in phase I clinical trials as an immunotoxin; the isolation of similar tumor-specific antibodies is an on-going project. Technical improvements have streamlined the morphologic hybridoma screening processing time to less than two days, allowing primary screening on cultured cells and secondary screening on tissue samples with the same initial 100 µL supernatant sample. 2) We have studied aspects of multidrug-resistance of human tumors in cultured cells and in tissues. We have localized P170, the product of the human MDR1 gene, in normal tissues using monoclonal antibody MRK16, and in addition to its location in the GI tract, adrenal, kidney and liver, we have also found it in capillaries in the central nervous system. We have studied the reactivity of monoclonal antibody C219 that reacts with an epitope of P170 present on the inner surface of the plasma membrane, and found that this antibody cross-reacts with other proteins in some tissues, such as the heavy chain of cardiac and slow-twitch muscle myosin, in human, monkey, and rat tissues. We have also examined the expression of human P170 introduced through a viral vector in MDCK cells and found that P170 is expressed in a polarized fashion on the apical surface of these cells, similar to its distribution in normal kidney proximal tubules. 3) In other studies, we have examined the effects of microinjected protein kinase C on cell morphology; we have localized the E5 oncogene product from papilloma virus in cultured cells; we have also localized monoclonal antibodies of nuclear pore carbohydrate epitopes using immunocytochemistry and microinjected lectins; and we have localized P58, a major thyroid hormone binding protein, to the cytosol of rapidly growing cells.

Other Professional Personnel:

I. Pastan	Chief, Laboratory of Molecular Biology	NCI
D. FitzGerald	Microbiologist	LMB, NCI
M. M. Gottesman	Chief, Molecular Cell Genetics Section	LMB, NCI
S.-y. Cheng	Research Chemist	LMB, NCI
F. Thiebaut	Visiting Fellow	LMB, NCI
J. A. Hanover	Senior Investigator	LMB, NCI
C. R.Schlegel	Chief, CRTS	LTVB, NCI
P. Blumberg	Chief, MMTPS	LCCTP, NCI

Major Findings:

Neoplastic transformation produces many changes in cell function, some of which are likely to be expressed as altered surface antigens. We have designed methods to isolate monoclonal antibodies to cell surface antigens present on human ovarian cancer cells. The goal of these studies is to isolate antibodies that are relatively tumor-specific for diagnostic as well as therapeutic purposes using immunotoxins constructed with these antibodies. Morphologic methods have provided extremely sensitive and precise techniques to isolate these antibodies and characterize their epitopes. We previously isolated a relatively tumor-specific antibody, OVB3, which is now undergoing phase I clinical trials as an immunotoxin in conjugation with Pseudomonas exotoxin. To make screening of hybridoma clones more efficient, we developed a screening chamber for immunofluorescence primary screening and a special low-volume incubation device for peroxidase labeling of tissue sections. These methods allow screening of supernatants within two days using the same small volume (~100 µL) sample. Using these new methods, we can conduct sequential fusions at a much faster rate, yet still evaluate normal tissue and tumor reactivity at high sensitivity. The same strategy is useful in screening antibodies to known surface proteins, such as P170.

Multidrug-resistant cells that express P170, the multidrug-resistance efflux pump protein, have been examined for the distribution of this protein in both culture and in intact tissues and tumors. Our previous studies using monoclonal antibody MRK16, that reacts selectively with human P170, showed high levels in jejunum, colon, kidney proximal tubules, the biliary face of hepatocytes and adrenal cortex and medulla. The lability of this protein prompted us to examine other tissues more carefully using multiple samples. We also evaluated a different antibody, C219, that reacts with a epitope in P170 present on the inside of the plasma membrane. The cross-reactivity of this antibody with other mammalian species allowed us to obtain reactive tissues that were extremely well preserved using monkey and rat tissues. While many of the sites reactive with C219 were in the same location as those reactive with MRK16, we found other sites of C219 reactivity, one of which was in the capilaries of the central nervous system. Since human brain samples are not always well-preserved, we re-evaluated multiple samples to see if MRK16 could be found to react with any capillaries in the human CNS; we found clear examples of this reactivity, suggesting that P170 is likely to be present in CNS capillaries. This result suggests caution in the design of immunotoxins directed to this protein for use in human therapy. Another result of these studies showed that C219 reacts with cardiac and skeletal muscle in rat and human samples, whereas, MRK16 and some

other anti-P170 antibodies do not. The protein responsible for this cross-reaction appears to be the heavy chain of cardiac and slow-twitch skeletal muscle myosin. This suggests caution in the use of C219 as a screening antibody for evaluation of P170 expression in tumor samples and normal tissues. In another study, we examined the display of P170 in MDCK cells in which the human MDR1 gene had been introduced using a viral vector. The results of this study showed that P170 was selectively placed on the apical surface of these cells in culture, analogous to its normal display in hepatocytes and cells of the proximal tubule of the kidney. This shows that the MDR1 gene is likely to contain the sequence information necessary to direct proteins to the apical cell surface in polarized epithelia. Preliminary experiments using these MDR-MDCK cells have suggested that these cells show polarized pumping of drugs such as daunomycin from base to apex when cultured on a filter support.

In other studies, we have collaborated with P. Blumberg and co-workers to examine the effects of protein kinase C and its catalytic fragment on the morphology of cells microinjected with these purified enzymes. Preliminary results suggest that these injected enzymes show activities which induce rapid changes in cell morphology somewhat similar to the changes seen when endogenous protein kinase C is activated using tumor promoters (phorbol esters).

In another study in collaboration with C. R. Schlegel, we have localized the E5 protein of bovine papilloma virus introduced into insect cells and into mouse cells using different vectors with affinity-purified antibodies to a synthetic peptide derived from the known sequence of the C-terminus of this small E5 peptide. Unexpectedly, the carboxy-terminal end of this hydrophobic peptide was found on the external face of the plasma membrane and on the lumenal surface of Golgi membranes using electron microscopic and immunofluorescence immunocytochemistry. The significance of this orientation of this trans-membrane peptide in transformation mediated by the E5 peptide of BPV is not yet clear.

In collaboration with J. A. Hanover, we have shown the association of certain monoclonal antibodies and labeled lectins with the nuclear pores of cells using both immunocytochemistry and microinjection methods. These studies demonstrate the presence of specific carbohydrate structures associated with nuclear pores that may relate to the function of nuclear pores in the targeting of cytosolic components to the nucleus.

In collaboration with S.-y. Cheng, we have localized P58, a thyroid hormone binding protein, to the cytosol of cells using immunocytochemistry with both light and electron microscopy. The evaluation of tumor and normal tissue distributions of this protein are underway. Preliminary results suggest a correlation of expression of P58 with rapid growth of cells.

Publications:

FitzGerald DJ, Bjorn MJ, Ferris RJ, Winkelhake JL, Frankel AE, Hamilton TC,Ozols RF, Willingham MC, Pastan I. Anti-tumor activity of an immunotoxin in a nude mouse model of human ovarian cancer, Cancer Res 1987;47:1407-10.

Cryz SJ, Furer E, Sadoff JC, Germanier R, Pastan I, Willingham MC, FitzGerald DJP. The use of Pseudomonas aeruginosa toxin A in the construction of conjugate vaccines and immunotoxins, Rev Infect Dis 1987;9:S644-9.

Willingham MC, Richert ND, Rutherford AV. A novel fibrillar structure in cultured cells detected by a monoclonal antibody, Exp Cell Res 1987;171:284-95.

Kitagawa S, Willingham MC, Pastan I, Cheng S-y. Thyroid hromone action: induction of morphological changes and stimulation of cell growth in rat pituitary tumor GH_3 cells, Endocrinol 1987;120:2591-2596.

Harris HW, Murhpy HR, Willingham MC, Handler JS. Isolation and characterization of specialized regions of toad urinary bladder apical membrane involved inthe water permeability response to antidiuretic hormone, J Membrane Biol 1987;96:175-86.

Willingham MC, Richert ND, Cornwell MM, Tsuruo T, Hamada H, Gottesman MM, Pastan I. Immunocytochemical localization of P170 at the plasma membrane of multidrug-resistant human cells, J Histochem Cytochem 1987;35:1451-6.

Gottesman MM, Pastan I, Fojo AT, Shen D-w, Ueda K, Clark DP, Cardarelli CO, Richert ND, Willingham MC, Cornwell MM, Chin JE, Chen C-j, Roninson IG. Expression, amplification and transfer of DNA sequences associated with mutidrug-resistance. In: Tew K, Wooley P, eds. Mechanisms of drug resistance in neoplastic cells. Orlando: Academic Press, 1987;243-57.

Fojo A, Cornwell MM, Cardarelli C, Clark DP, Richert N, Shen D-w, Ueda I, Willingham M, Gottesman MM, Pastan I. Molecular biology of drug resistance, Breast Cancer Res Treat 1987;9:5-16.

Willingham MC, FitzGerald DJ, Pastan I. Pseudomonas exotoxin coupled to a monoclonal antibody against ovarian cancer inhibits the growth of human ovarian cancer cells in a mouse model, Proc Natl Acad Sci USA 1987;84:2474-8.

FitzGerald DJ, Willingham MC, Cardarelli CO, Hamada H, Tsuruo T, Gottesman MM, Pastan I. A monoclonal antibody-Pseudomonas-toxin conjugate that specifically kills multidrug-resistant cells, Proc Natl Acad Sci USA 1987;84:4288-92.

Hanover JA, Cohen CK, Willingham MC, Hanover JA. A monoclonal antibody against a family of nuclear pore proteins (nucleoporins): O-linked N-acetyl-glucosamine is attached to proteins of the nuclear pore: evidence for cytoplasmic glycosylation, J Biol Chem 1987;262:9887-84.

Willingham MC, Rutherford AV, Cheng S-y. Immunohistochemical localization of a thyroid hormone-binding protein (P55) in human tissues, J. Histochem Cytochem 1987;35:1043-6.

Park MK, D'Onofrio M, Willingham MC, Hanover JA. A monoclonal antibody against a family of nuclear pore proteins (nucleoporins): O-linked N-acetyl-glucosamine is part of the immunodeterminant, Proc Natl Acad Sci USA 1987;84:6462-6.

Velu TJ, Beguinot L, Vass WC, Willingham MC, Merlino GT, Pastan I, Lowy DR. Epidermal growth factor-dependent transformation by a human EGF receptor proto-oncogene, Science 1987;238:1408-10.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues, Proc Natl Acad Sci USA 1987;84:7735-8.

Goldenthal KL, Hedman K, Chen JW, August JT, Vihko P, Pastan I, Willingham MC. Prelysosomal divergence of alpha$_2$-macroglobulin and transferrin: a kinetic study using a monoclonal antibody against a lysosomal membrane glycoprotein (LAMP-1), J Histochem Cytochem 1988;36:391-400.

Pirker R, FitzGerald, DJP, Willingham MC, Pastan I. Enhancement of the activity of immunotoxins made with either ricin A chain or Pseudomonas exotoxin, Cancer Res 1988; in press.

FitzGerald DJ, Willingham MC, Pastan I. Pseudomonas exotoxin - immunotoxins. In: Frankel A, ed. Immunotoxins. Martinus Nijhoff B.V., 1988; in press.

Gottesman MM, Willingham MC, Thiebaut F, Pastan I. Expression of the MDR1 gene in normal human tissues. In: Roninson I, ed. Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells. New York, Plenum Publishing Corp, 1988; in press.

Padmanabhan R, Corsico C, Howard T, Holter W, Fordis M, Willingham MC, Howard BH. Purification of transiently transfected cells by magnetic affinity cell sorting, Anal Biochem 1988; in press.

Gallo M, Chaudhary V, FitzGerald DJP, Willinghman MC, Pastan I. Cloning and expression of the heavy chain variable region of antibody OVB3 that reacts with human ovarian cancer, J Immunol 1988; in press.

Pastan I, Gottesman MM, Ueda, K, Lovelace E, Rutherford AV, Willingham MC. A retrovirus carrying a MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells, Proc Natl Acad Sci USA 1988; in press.

Wolff B, Willingham MC, Hanover JA. Nuclear protein import: specificity for transport across the nuclear pore, Exp Cell Res 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08011-14 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 charactars 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:	M. Obara'	Visiting Fellow	LMB, NCI
	M. S. Kang	Biotechnology Fellow	LMB, NCI
	S. Shinagawa	Guest Researcher	LMB, NCI
	M. J. Humphries	Guest Researcher	LMB, NCI
	K. Olden	Guest Reseacher	LMB, NCI
	Y. Yasuda	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 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.4	3.7	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.)*

Fibronectin is a major cell surface and extracellular matrix glycoprotein involved in cell adhesion and migration. A synthetic peptide containing the key fibronectin adhesive recognition sequence Gly-Arg-Gly-Asp-Ser administered intravenously substantially enhanced survival of mice challenged concurrently with melanoma cells in an experimental metastasis assay. Since renal clearance of the peptide was found to be very rapid, conjugation of a peptide derivative to a carrier is being tested as a means of increasing its time in the circulation. In addition to this original peptide recognition sequence, fibronectin was shown to contain a second binding site that synergizes with it to produce full adhesive affinity and efficient transmembrane effects of the peptide on cytoskeletal organization. Mutants produced by site-directed mutagenesis or deletion of each site displayed >96% losses of activity, and the two types of mutants synergized in vitro for adhesion and promoting organization of actin microfilament bundles. Studies of this region may yield a novel class of inhibitor of fibronectin function. Two other cell adhesion sequences are present in only certain fibronectin molecules due to alternative mRNA splicing of another domain. Besides melanoma cells, embryonic cells from the peripheral nervous system were found to use this cell-type specific domain, which may be recognized only by cells derived originally from the neural crest. The Gly-Arg-Gly-Asp-Ser peptide and related analogues inhibited adhesion of embryonic cells involved in somite and heart formation. However, this peptide had unusual effects on segmental plate cells, which are the precursors of somites. The peptide substantially induced cell-to-cell aggregation, suggesting that it may regulate expression of a cell-cell adhesion system.

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

Major Findings:

Fibronectin is a major cell surface and extracellular matrix glycoprotein involved in cell adhesion and migration. We have explored the structural basis of its activities, focusing on the peptide sequences essential for its interactions with different cell types and on its role in normal and malignant cell adhesion, migration, and invasion. These studies have led to the development of specific inhibitors that block important biological events in vivo, including experimental metastasis.

Synthetic peptide inhibitors of specific fibronectin adhesive functions are being developed and characterized. A synthetic peptide comprising the key fibronectin adhesive recognition sequence Gly-Arg-Gly-Asp-Ser (GRGDS) inhibits experimental metastasis in the B16-F10 melanoma assay. Animals treated with GRGDS showed substantially increased survival after tumor cell challenge, e.g., 8/8 treated mice survived > 15 months, while 8/8 untreated mice died in less than 2 months; similar protection was observed in two other, short-term experiments. However, assays of the activity of GRGDS and related synthetic peptides in cell adhesion assays in vitro revealed a decrease of biological activity as large as two orders of magnitude in comparison with intact fibronectin. Moreover, after intravenous injection, levels of GRGDS in blood fell very rapidly due to direct renal clearance without metabolic alteration, with a half-time of 8 minutes. Coupling the peptide to a rabbit IgG carrier substantially prolonged survival in the circulation. Preliminary experiments indicate that such conjugated molecules are also substantially more active on a molar basis at inhibiting cell adhesion compared with the free peptide. Alternative non-immunogenic conjugates are being developed, and the activity of various peptide-conjugated carriers are being tested for metastasis inhibition.

To understand why synthetic peptides do not reproduce the full activity of fibronectin, we analyzed the molecular basis of fibronectin function in more detail. Using site-directed mutagensis and deletion analysis, we found that fibronectin interacts with the cell surface by means of a bipartite domain requiring two synergistic sites. Site-specific deletion of the previously identified adhesive recognition sequence Arg-Gly-Asp-Ser or even site-specific mutation of the Asp to a closely related Glu residue decreased adhesive activity of fibronectin fusion proteins expressed in E. coli by > 97%. A second key functional site over 0.5 kb away was mapped by deletion mutagenesis. The latter mutants also displayed a > 96% loss of activity, indicating cooperativity between the sites, a conclusion supported by in vitro trans complementation assays. In addition, effective actin microfilament bundle formation was also found to be dependent on the combined function of both sites. Thus, both fibroblast adhesion and a major transmembrane response to fibronectin involve two synergistic sites, each of major quantitative importance. The key amino acids in this novel synergistic site identified by deletion mutagenesis are being mapped further by stepwise oligonucleotide-directed deletion and synthetic peptide analysis in order to develop novel inhibitors of fibronectin-mediated adhesion and migration.

Fibronectin also contains sequences that are present in only certain fibronectin molecules due to processing by alternative splicing of precursor mRNA. We showed that two alternatively spliced sequences were cell-type specific adhesion

sites, and developed synthetic peptide inhibitors of each site as well as peptide conjugates that could directly mediate adhesion. We found that besides the originally identified melanoma cell targets, embryonic neurons from both sympathetic and dorsal root ganglia, as well as their neural crest cell precursors, were able to adhere to the CS1 spliced site. The adhesive activity of this site was found to be directly additive to activity expressed by the other, general cell adhesive domain present in all fibronectins. These results suggest that this alternatively spliced domain is recognized specifically by cells of the embryonic neural crest lineage, but not by other cells; this cell-type specificity provides a possible regulatory mechanism for neuronal development dependent on the local expression of alternatively spliced sites. The existence of two alternatively spliced and two synergistic recognition sites in fibronectin that cooperate with each other to produce full adhesive activity and specificity provides a simple mechanism for cell adhesive recognition based on cooperative interactions that may be a model for other adhesion systems.

The role of fibronectin adhesive recognition sites in embryonic development was explored further in the processes of formation of somites and the heart. The GRGDS sequence appears to be involved in both events, since synthetic peptides containing this sequence blocked adhesion of cells from both systems. However, the effects on cell-to-substrate versus cell-to-cell adhesion varied depending on the developmental system. The most interesting finding was that GRGDS treatment induced striking increases in cell-to-cell adhesion of segmental plate cells, the transient precursors of somites; the effect was specific to this cell type. This unexpected finding suggests that the GRGDS peptide can trigger the function of a cell-cell adhesion system, e.g., N-cadherin or N-CAM. It will, therefore, be of interest to measure directly the levels of such cell-cell adhesion molecules after GRGDS treatment, since this effect may be the first example of the control of cell-cell adhesion molecule expression by an adhesive recognition signal.

Publications:

Humphries MJ, Yamada KM, Olden K. Investigation of the biological effects of anti-adhesive synthetic peptides which inhibit experimental metastasis of B16-F10 murine melanoma cells, J Clin Invest 1988;81:782-90.

Humphries MJ, Obara M, Olden K, Yamada KM. Role of fibronectin in adhesion, migration, and metastasis, Cancer Invest 1988; in press.

Humphries MJ, Yasuda Y, Olden K, Yamada KM. The cell interaction sites of fibronectin in tumor metastasis, CIBA Symp 1988; in press.

Obara M, Kang MS, Rocher-Dufour S, Kornblihtt A, Thiery JP, Yamada KM. Expression of the cell binding domain of human fibronectin in E. coli: Identification of sequences promoting full to minimal adhesive function, FEBS Lett 1987;213:261-4.

Obara M, Kang MS, Yamada KM. Site-directed mutagenesis of the cell-binding domain of human fibronectin: Separable, synergistic sties mediate adhesive function, Cell 1988; in press.

Humphries MJ, Komoriya A, Akiyama SK, Olden K, Yamada KM. Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion, J Biol Chem 1987;262:6886-92.

Humphries MJ, Akyama SK, Komoriya A, Olden K, Yamada KM. Neurite extension of chicken peripheral nervous system neurons on fibronectin: Relative importance of specific adhesion sites in the central cell-binding domain and the alternatively-spliced type III connecting segment, J Cell Biol 1988;106:1289-97.

Lash JW, Linask KK, Yamada KM. Synthetic peptides that mimic the adhesive recognition signal of fibronectin: Differential effects on cell-cell and cell-substratum adhesion in embryonic chick cells, Dev Biol 1987;123:411-20.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08705-12 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

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
Other:	S. Goldenberg	Research Chemist	LMB, NCI
	M. Chapman	Research Biologist	LMB, NCI
	R. Fleischmann	Staff Fellow	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	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.)*

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 the manner in which cyclic AMP regulates cell growth and gene expression. The mechanism of cAMP action on CHO cells has been studied by isolating CHO mutants resistant to growth inhibition by cAMP. These mutants have defective cAMP dependent protein kinases with either altered regulatory (RI) or catalytic (C) subunits. DNA from cells carrying dominant cAMP-resistant defects can be used to transfer the cAMP-resistance phenotype to sensitive cells. Cosmid clones carrying the CHO RI subunit from cAMP resistant cells have been isolated. Sequence analysis indicates that one of these clones, pcosRI has the coding sequence for the CHO RI subunit. The exon-intron structure of the RI gene is complex; the region encoding the cAMP-binding site at the 3' end of the gene is composed of several different exons. The regulation of expression of cAMP-responsive genes is being studied using wild type, cAMP dependent protein kinase mutants, and transferent CHO cell lines expressing the mutant kinase phenotype. We have demonstrated that for the rat tyrosine amino transferase (TAT) gene promoter an intact cAMP dependent protein kinase system is necessary for the regulation of cAMP responsive gene expression.

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

Major Findings:

1. We have prepared cosmid libraries of DNA From dominant CHO mutants carrying altered regulatory (RI) and catalytic (C) subunits of cAMP dependent protein kinases. Using an RI cDNA probe (gift of S. McKnight), we have isolated cosmid clones from these libraries for the RI genes. Sequence analysis of one RI genomic clone, pcosRI, confirms that it contains the coding sequence of CHO RI. The RI gene has at least seven coding exons, of which four have been completely sequenced. Two exons are within the cAMP binding domain at the 3' end of the gene.

2. We have used several CHO cAMP dependent protein kinase mutants and transferents to study the regulation of the expression of genes by cAMP in order to determine whether cAMP-stimulated gene expression requires an intact protein kinase system. Wild-type and mutant CHO cells were transfected with a variety of cloned DNAs containing promoter regions linked to the bacterial chloramphenicol acetyl transferase (CAT) gene. Tyrosine amino transferase (TAT)-CAT, RSV-CAT, pSV_2-CAT, α_2-collagen-CAT and transferrin receptor-CAT constructions all expressed CAT activity which was stimulated to varying extents by cAMP. This cAMP-mediated stimulation was not seen in any of the CHO mutants with altered cAMP dependent protein kinase activity, indicating that this kinase is an essential intermediate for this effect of cAMP.

3. The regulation of the rat TAT gene was studied in most detail. Expression of the rat TAT gene is increased in the presence of cAMP and agents which increase adenylate cyclase activity such as cholera toxin. Wild type CHO cells, three classes of cAMP dependent protein kinase mutants, and a transferent CHO cell line were transfected with a cloned 2.5 kb promoter fragment from the rat TAT gene linked to the bacterial chloramphenicol acetyl transferase (CAT). The TAT-CAT construction expressed CAT activity in all the CHO cell lines and was stimulated three- to four-fold by cAMP in the wild type cell line. This cAMP-mediated stimulation was not seen in any of the CHO mutants or the transferent with altered cAMP dependent protein kinase activity, indicating that this kinase is an essential intermediate for the effect of cAMP on TAT gene expression.

4. Some cAMP dependent protein kinase mutants are supersensitive to natural product cytotoxic drugs such as colchicine, vinblastine, adriamycin, and puromycin. Revertant cells which have recovered normal levels of drug-resistance have lost their protein kinase mutant phenotype. This result suggests that the expression of resistance to multiple drugs can be modulated by cAMP dependent protein kinase, perhaps through a mechanism involving the multidrug transporter (MDR1 gene product).

Publications:

Abraham I, Hunter R, Sampson K, Smith S, Gottesman MM, Mayo J. cAMP dependent protein kinase regulates sensitivity of cells to multiple drugs, Mol Cell Biol 1987;1:3098-106.

Gottesman MM. Chinese hamster ovary cells, Methods Enzymol 1987;151:3-8.

Gottesman MM. Drug-resistant mutants: Selection and dominance analysis, Methods Enzymol 1987;151:113-21.

Gottesman MM. Strategies for isolation of mutant genes, Methods Enzymol 1987;151:329-332.

Fleischmann R, McCormick M, Howard BH. Preparation of a genomic cosmid library, Methods Enzymol 1987;151:405-16.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08710-12 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

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 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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 molecular mechanisms involved in DNA replication and its regulation are being studied biochemically. To study properties of origin specific initiation of replication in vitro, I have developed an enzyme system that replicates exogenously added plasmid DNA containing the initiation origin of bacteriophage P1. The system requires the addition of the P1 replication protein, RepA, a partially purified protein fraction to supply E. coli replication proteins, ATP and an ATP regenerating system, dNTPs, rNTPs, Mg^{+2}, buffer and polyvinyl alcohol. The specific E. coli replication proteins required include RNA polymerase, dnaA, dnaB, dnaC, dnaG, DNA pol III and DNA elongation accessory proteins and DNA gyrase. I have been studying the interaction of RepA and dnaA proteins with the P1 origin DNA. I have also been characterizing by deletion analysis the minimal DNA site required for initiation of replication. Since P1 normally exists stably as a unit copy plasmid, this system is being used to study the molecular mechanisms involved in the regulation of a stringently controlled replicon. A plasmid control locus, incA, is required for this regulated replication in addition to the origin region. It has been proposed that RepA is rate limiting for replication and that the role of incA is to sequester the initiator and thus reduce replication. This proposal appears inconsistent with the observation that RepA is autoregulated, since protein lost by sequestration should be replenished. A resolution of this autoregulation-sequestration paradox is possible if the sequestered RepA, unavailable for replication, is still available for promoter repression. In collaboration with D. Chattoraj (LB, NCI, NIH), we have shown that the RepA protein binds to incA and simultaneously to the repA promoter in the origin region, causing the intervening DNA to loop. DNA looping very likely could provide the mechanism by which RepA bound to incA might exert repression.

Major Findings:

I have been studying the in vitro replication of mini P1 DNA. The system I have developed consists of plasmid DNA containing the P1 origin of replication, purified RepA, the P1 initiation protein, a partially purified protein fraction from uninfected E. coli cells to supply E. coli replication proteins, 4 dNTPs, 4 rNTPs, buffer, Mg^{+2}, an ATP regenerating system and polyvinyl alcohol. I have used inhibitors, antibodies and complementation tests to show that dnaA, dnaB, dnaC, RNA polymerase and DNA gyrase are required. Genetic studies have indentified the origin to be a 250 bp piece of P1 DNA containing five repeats of a 19 base pair sequence to which the RepA protein binds. To the left of the RepA binding sites is an adenine and thymine rich region that contains four direct repeats of a 9 base pair sequence. Further to the left are two direct repeats of the dnaA protein binding site. I have found that dnaA protein binds to this region and that the ADP form of dnaA is active in initiation. In contrast, the replication of plasmid DNAs containing the E. coli origin specifically require the ATP form of dnaA. In collaboration with K. McKenney (National Bureau of Standards, Gaithersburg, MD), we have cloned the P1 region in M13, constructed and sequenced deletions of both the right and the left ends and tested RF DNA of these deletion phage for their ability to support RepA dependent replication in vitro. We have found that P1 DNA to the left of the dnaA boxes could be deleted without loss of origin activity, but deletion of one and one-half dnaA boxes resulted in total loss of activity. Thus, dnaA binding sites as well as dnaA protein are required for the initiation reaction. On the right, two and one-half RepA boxes could be deleted without loss of activity. However, deletion of four and one-half boxes resulted in loss of activity. We are determining more exactly the limits of the minimal ori region and will test the deletions for origin activity in vivo.

This system provides, for the first time, a means for studying the regulation of a stringently controlled replicon, since P1 normally exists as a unit copy plasmid. A control region, incA, that is located immediately downstream and to the right of the repA gene, is required for this regulated replication. The repA gene itself is to the right of the origin with its promoter situated within the RepA binding sites in the origin. The control region contains nine 19 bp repeats similar to those in the origin. The RepA protein binds equally well to the origin and control repeats. Thus the lower copy number of plasmids containing the control region could be simply explained by a sequestration of RepA by the repeat units in the control region. However, the repA gene is autoregulated, and its expression would be expected to increase as its product is sequestered. In collaboration with D. Chattoraj (LB, NCI, NIH), we have electron microscopic evidence for RepA dependent DNA looping between the origin region and the control region. Thus RepA bound to the control region might exert repression by looping back and making a second protein-DNA contact with a repeat in the promoter region. In addition to repressing the repA gene, this specific RepA dependent DNA looping could also regulate replication directly by inhibiting initiation complex formation at the origin. We are currently testing this using the in vitro replication system.

Publications:

Wickner S, Chattoraj D. Replication of mini P1 plasmid DNA in vitro requires two initiation proteins. The products of P1 repA and E. coli dnaA, Proc Natl Acad Sci USA 1987;84:3668-72.

Wickner S, McKenney K. Deletion analysis of the DNA sequence required for the in vitro initiation of replication of bacteriophage λ, J Biol Chem 1987;262:13163-7.

Chattoraj DK, Mason RJ, Wickner SH. Mini-P1 plasmid replication: The autoregulation-sequestration paradox, Cell 1988;52:551-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08714-11 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Bacterial Functions Involved in Cell Growth Control

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

PI:	S. Gottesman	Chief, Biochemical Genetics Section	LMB, NCI
Other:	M. Maurizi	Senior Fellow	LMB, NCI
	J. Trempy	Guest Researcher	LMB, NCI
	W. Clark	Research Chemist	LMB, NCI
	N. Trun	IRTA Fellow	LMB, NCI
	V. Stout	Staff Fellow	LMB, NCI
	S. H. Kim	Visiting Fellow	LMB, NCI

COOPERATING UNITS *(if any)*
Laboratory of Genetics, Division of Cancer Biology and Diagnosis

S. Rudikoff	LG, NCI
J. Pumphrey	LG, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.8	5.8	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 and independent protein turnover. E. coli lon mutants are defective in cell division regulation after DNA damage, and overproduce capsular polysaccharide. We have previously shown that an unstable positive regulator of capsule synthesis, RcsA, is stabilized in lon mutants. The rcsA function seems to be common to a number of gram-negative prokaryotes, suggesting a common mechanism for regulating different capsules in these strains. RcsC, a negative regulator of capsule synthesis, is a membrane protein which may act as a sensor for environmental signals controlling capsule synthesis. The gene for the ATP-binding subunit of a new two component ATP-dependent protease has been isolated, and chromosomal mutations in the gene, called clpA, have been generated. While cells devoid of clpA are healthy, the Clp protease seems to act as a back-up to lon in degrading abnormal proteins. A third protease function has been identified, and the gene responsible has been mapped to 56 minutes on the E. coli chromosome.

GPO 914-918

Major Findings:

1. RcsB, a positive regulator of capsule synthesis, plays an unknown role in mediating the synthesis of capsule, but is absolutely essential. A variety of genetic experiments suggest that RcsB may interact with the negative regulator, RcsC. rcsB-lac protein fusions were created in vitro; the isolation of the rcsB-lacZ protein has allowed us to raise antibodies to the RcsB protein. An examination of the expression of these fusions in a variety of regulatory mutant strains has suggested that the synthesis of rcsB does not change dramatically in response to any of our regulatory mutations. Therefore, if RcsB is responsible for modulating capsule synthesis, its activity rather than its synthesis may be changed by protein modification. The rcsC product is unable to be fused to β-galactosidase, presumably because such fusions are lethal. Such lethality is characteristic of fusions to many membrane proteins. Much stronger evidence of the membrane localization of rcsC is provided by the isolation of functional protein fusions of alkaline phosphatase to rcsC. In these fusions, phoA is only functional if present in the periplasm; localization to the periplasm depends on information in the rcsC protein. In addition, these fusions should allow the isolation of the fusion protein. The genetic properties of rcsC and rcsB suggest that they may act as a sensor-positive regulator pair, possibly with homology to other such pairs which have been identified in E. coli.

2. We have used a biochemical approach to identify one of the structural genes for a new, two-component ATP-dependent protease from E. coli. In addition, we have developed the materials to allow identification of the other, unlinked component. Using antibodies to the purified ATP-binding subunit of the protease, we identified lambda clones carrying the gene, which we call clpA. Insertional mutagenesis of the phage clone and transfer of mutations from the phage to the bacterial chromosome allowed the mapping of the gene to the 19' region of the chromosome. In addition, insertions within the chromosomal clpA gene allowed us to demonstrate that clpA is dispensable for E. coli. clpA lon double mutants have decreased turnover of abnormal proteins compared to the lon parent, suggesting that clpA functions at least partially as a second, less efficient system for degradation of abnormal proteins. ClpA, unlike Lon, is not a heat shock protein. We can now use the clpA mutations to look for specific in vivo Clp substrates. We have also isolated clpA-lacZ protein fusions, which will simplify the examination of regulation of clpA expression in cells.

3. A genetic approach to identifying E. coli proteases has yielded a function which, when present on multi-copy plasmids, can suppress the two major lon phenotypes, UV sensitivity and capsule overproduction. The function, which we have called alp, does not appear to be essential for cell growth. Degradation of the unstable cell division inhibitor, SulA, which is decreased in lon mutants, is increased when plasmids carrying alp+ are present in cells, but not when the alp gene on the plasmid is inactivated by insertions. Cells carrying the multi-copy alp plasmid grow poorly and accumulate mutations within the plasmid; we had previously observed similar problems with cells carrying the multi-copy lon plasmid. The poor growth suggests that cellular substrates for lon and alp necessary for cell growth are being degraded by the high levels of the protease in these cells. An analysis of the effect of alp mutations and the alp plasmid on cell growth should help us identify natural substrates for the alp protease system.

Publications:

Torres-Cabassa A, Gottesman S, Frederick RD, Dolph PJ, Coplin DL. Control of extracellular polysaccharide synthesis in Erwinia stewartii and Escherichia coli K-12: A common regulatory function, J Bacteriol 1987;169:981-9.

Brill JA, Quinlan-Walshe C, Gottesman S. Fine-structure mapping and identification of two regulators of capsule synthesis in Escherichia coli K-12, J Bacterial 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08715-10 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Synthesis and Function of a Transformation-Dependent Secreted Lysosomal Protease

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
Other:	S. Goldenberg	Research Biologist	LMB, NCI
	M. Chapman	Research Biologist	LMB, NCI
	B. Troen	Medical Staff Fellow	LMB, NCI
	S. Kane	Guest Researcher	LMB, NCI
	S. Smith	Guest Researcher	LMB, NCI
	A. Salminen	Visiting Fellow	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	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.)*

Cultured mouse fibroblasts which are malignantly transformed or treated with TPA or growth factors such as PDGF synthesize and secrete the 39,000 Mr precursor to cathepsin L in large amounts. This purified procathepsin L, originally called MEP for major excreted 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 with a lysosomal localization. Overproduction of cloned cathepsin L in a nontransformed cell results in secretion of this lysosomal enzyme. Secreted procathepsin L can bind to the mannose 6-phosphate receptor of many cells and be endocytosed and processed intracellularly. In antigen presenting cells, procathepsin L uptake reduces the efficiency of antigen presentation, thereby interfering with immune response. Transformation, TPA and PDGF stimulate procathepsin L synthesis by increasing levels of procathepsin L specific mRNA transcription. We have cloned a functional procathepsin L gene from the mouse and have identified and sequenced the 5' flanking region which acts as a promoter in CAT constructions. A human procathepsin L cDNA has also been cloned and sequenced. Human procathepsin L mRNA has been detected in all normal human tissues analyzed with highest levels in liver, spleen, kidney, and many malignant tumors. Recombinant human procathepsin L produced in bacteria is enzymatically active.

Major Findings:

1. The major excreted protein (MEP) of transformed mouse fibroblasts is procathepsin L based on its substrate specificity, inhibition specificity, and its deduced amino acid sequence. It is a broad spectrum acid protease with a pH optimum of 5.5.

2. Procathepsin L added to antigen-presenting cells which process and present pigeon cytochrome C reduces the efficiency of antigen presentation. The mechanism of this effect involves endocytosis of procathepsin L via mannose 6-phosphate receptors. Within the cell, cathepsin L protease activity is presumed to destroy the antigen. This result suggests that one function of MEP may be to alter the immune response of tumor bearing hosts.

3. A cloned cDNA encoding mouse procathepsin L recognizes a 1.6 - 1.8 kb mRNA in nontransformed and transformed mouse cells. A human MEP analog has also been cloned and recognizes a 1.6 - 1.8 kb mRNA in all human cells tested. The highest levels of procathepsin L mRNA are found in liver, spleen, and kidney. Many cancers have elevated levels of procathepsin L mRNA. Complete coding sequences for mouse and human MEP have been obtained. The deduced amino acid sequences have been used to determine the cleavage sites in preprocathepsin L to yield the secreted procathepsin L and the processed lysosomal 29 kDa and 20 kDa forms.

4. The mouse MEP (procathepsin L) gene has been cloned. It is contained on a 14 kb segment of the cosmid pcosMMEP. pcosMMEP can be transferred to nontransformed monkey CV-1 or transformed human A431 cells where the mouse form of MEP is expressed. We have mapped the site for initiation of transcription. A 1 kb fragment 5' to this initiation site contains typical sequence elements of a promoter and acts as a promoter in CAT constructions.

5. Cloned mouse and human procathepsin L can be transferred to mouse cells and expressed at high levels using the pHaMDR1 amplification system. High levels of procathepsin L do not produce a transformed phenotype in NIH 3T3 cells, but they do result in secretion of procathepsin L, indicating that overproduction of this lysosomal protein is sufficient to result in its secretion.

6. Recombinant human procathepsin L has been produced in large quantities in E. coli. Yields of approximately 0.5g/10L culture have been obtained. The recombinant enzyme is active after solubilization in urea and reduction/oxidation in glutathione above pH 10.

Publications:

Troen BR, Gal S, Gottesman MM. Sequence and expression of the cDNA for MEP (major excreted protein), a transformation-regulated secreted cathepsin, Biochem J 1987;246:731-5.

Troen BR, Ascherman D, Atlas D, Gottesman MM. Cloning and expression of the gene for the major excreted protein of transformed mouse fibroblasts, J Biol Chem 1988;263:254-61.

Mason RW, Gal S, Gottesman MM. The identification of the major excreted protein (MEP) from a transformed mouse fibroblast cell line as a catalytically active precursor form of cathepsin L, Biochem J 1988;248:449-54.

McCoy K, Gal S, Schwartz R, Gottesman MM. An acid protease secreted by transformed cells interferes with antigen processing, J Cell Biol 1988; in press.

Kane SE, Troen BR, Gal S, Ueda I, Pastan I, Gottesman MM. Use of a cloned multidrug-resistance gene for co-amplification and overproduction of MEP, a transformation-regulated secreted acid protease, Mol Cell Biol 1988; in press.

Gal S, Gottesman MM. Isolation and sequence of a cDNA for human procathepsin L, Biochem J 1988; in press.

Troen, BR. Colony screening of genomic cosmid libraries, Methods Enzymol 1987; 151:416-26.

Gal S. Replica plating and detection of antigen variants, Methods Enzymol 1987;151:104-12.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08717-10 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Functions, Structure, and Regulation 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
Other:	S. K. Akiyama	Guest Researcher	LMB, NCI
	S. S. Yamada	Guest Researcher	LMB, NCI
	H. Larjava	Visiting Fellow	LMB, NCI
	W.-T. Chen	Guest Researcher	LMB, NCI
	Y. Y. Yeh	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 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.6	3.6	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 space provided.)*

Cells interact with extracellular matrix molecules by specific receptors. We have characterized membrane binding molecules for fibronectin and collagen. The biosynthesis, regulation, and function of fibronectin receptors were characterized. The beta subunit of mouse and human receptors were found to be synthesized as smaller precursors that required a lengthy maturation period involving asparagine-linked oligosaccharides. Maturation resulted in an activation of fibronectin-binding function and secretion onto the cell surface. Levels of both alpha and beta subunits of the human fibronectin receptor were regulated by transforming growth factor β, which induced synthesis 20- to 40-fold. Fibronectin receptors on the cell surface were shown to exist in a transmembrane orientation. They are often diffuse on rapidly migrating embryonic cells and avian or human tumor cells, but clustered at adhesion sites of stationary cells. These distributions were shown to be independent of receptor phosphorylation. Roles for the fibronectin receptor were shown in embryonic amphibian gastrulation and human platelet function. Antibodies against the receptor blocked gastrulation and platelet adhesion to fibronectin. The receptor corresponds to platelet glycoprotein Ic-IIa, and is involved in activation-independent attachment of platelets to fibronectin-containing substrates. A protein that was decreased after malignant transformation was characterized biochemically. It is a globular monomer enriched in basic amino acids, with a unique amino-terminal 36-residue sequence containing a novel 7-mer tandem repeat unrelated to other heat shock proteins. Heat shock induces levels of mRNA as detected by *in vitro* translation and Northern blot analysis. Two putative sequential biosynthetic precursors of this hsp47 protein were identified, both of which were fully biologically active in binding to a collagenous ligand; this immediate functionality contrasts with the requirement for activation of the fibronectin receptor.

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

Major Findings:

The biosynthesis, regulation and function of cellular receptors for fibronectin were characterized. We raised monospecific or monoclonal antibodies against human, mouse, and chicken fibronectin receptors. The pathways of biosynthesis of mammalian and avian fibronectin receptors were found to differ substantially in the time required for maturation. The beta subunit of both mouse and human receptors was synthesized as a smaller inactive precursor that required a relatively slow maturation period of up to 20 hours involving an aspargine-linked oligosaccharide moiety which was sensitive to digestion by the enzyme endoglyosidase F. Maturation resulted in an activation of fibronectin-binding function and secretion onto the cell surface. The results indicate that this receptor is not initially active, and must undergo biosynthetic processing to achieve functional activity and cell surface expression; this requirement might provide a secondary means of regulating function besides the direct control of levels of the protein.

Regulation of the expression of both the alpha and beta subunits of the human fibronectin receptor, as well as of fibronectin itself, was found after treatment of fibroblasts by transforming growth factor β. Levels of fibronectin mRNA and protein biosynthesis were increased by as much as 30-fold after treatment with TGF-β1. Similarly, levels of fibronectin receptor synthesis were increased 20- to 40-fold. The induction of mRNA was blocked by actinomycin D, suggesting regulation at the level of transcription.

Once synthesized and expressed on the cell surface, fibronectin receptors were shown to exist in a transmembrane orientation using a novel immunocytochemical approach based on monoclonal antibodies directed against either extracellular or cytoplasmic domains of the protein and a staining protocol specific for the intracellular side of the plasma membrane. These fibronectin receptors can exist in either diffuse or clustered distributions. For example, the receptor was shown to be diffuse on a variety of avian or human tumor cells in vitro, as well as on rapidly migrating embryonic neural crest and somitic fibroblasts. In contrast, as the latter embryonic cells become stationary as they mature developmentally, the receptor becomes clustered. Although tyrosine phosphorylation of this receptor is a popular candidate for regulation of its function, no correlation could be found between receptor phosphorylation and its distribution or function. For example, even though tyrosine phosphorylation was substantially increased on Rous sarcoma virus-transformed receptors, similarly diffuse receptors on motile embryonic cells were not detectably phosphorylated; conversely, stationary embryonic cells showed substantial levels of receptor phosphorylation. These findings suggest that some other, as yet unidentified, mechanism of regulation affects receptor organization and function.

Additional collaborative studies have examined the roles of the fibronectin receptor in gastrulation and in platelet function. The receptor is present in amphibian embryos, and appears to play a critical role in the massive cell migratory movements of gastrulation. Anti-receptor antibodies in solution block the adhesion of ecto-mesodermal and endodermal cells to fibronectin, and the antibody when adsorbed itself onto substrates can mimic the adhesive activity of fibronectin. Microinjection of these antibodies into embryos blocks gastrulation, indicating a role in this important morphogenetic process.

Although the platelet receptor GP IIb-IIIa, an activation-dependent relative of the fibronectin receptor, was previously thought to be the only major fibronectin/fibrinogen/von Willebrand factor receptor of platelets, our anti-receptor antibodies from both avian and human receptors have identified substantial amounts of regular fibronectin receptor on platelets. It corresponds to the platelet component GP Ic-IIa, and it permits the attachment of platelets to extracellular matrices containing fibronectin without a requirement for prior activation.

We extended studies of a collagen-binding protein that we had found to be a novel heat-shock protein, now termed hsp47. This 47,000 dalton glycoprotein is decreased after malignant transformation of avian cells. We examined its biochemical properties, regulation, and requirements for function. It exists as a nearly globular monomer with an amino acid composition unusually enriched in basic amino acids and glycine, and with virtually no cysteine. Amino-terminal sequencing covering 36 residues revealed a single, novel sequence with an internal tandem repeat of Asp-Lys-Ala-Thr-Thr-Leu-Ala and Asp-Arg-Ser-Thr-Thr-Leu-Ala, which are not present in other proteins. Hsp47 appears to be unrelated to a variety of other heat-shock or collagen-binding proteins.

The induction of hsp47 expression by heat shock was shown to be inhibited by actinomycin D. Messenger RNAs purified from normal, heat-treated, and malignantly transformed chick embryo fibroblasts were analyzed in an in vitro translation system and by Northern blot analysis. Levels of mRNA were increased after heat shock and decreased after transformation. Two putative precursors of hps47 were identified using an in vitro translation/processing system and tunicamycin: one was a 42-kDA primary translation product and the second was a 41-kDa polypeptide lacking the signal peptide and carbohydrate moieties. In contrast to the fibronectin receptor and a number of other glycosylated binding proteins, both precursors of this protein appeared to be fully biologically active in binding to the collagen ligand. This collagen-binding protein shows the diversity of heat-shock protein function and of the mechanisms of receptor functional activation.

Publications:

Akiyama SK, Yamada KM. Biosynthesis and acquisition of biological activity of the fibronectin receptor, J Biol Chem 1987;262:17536-42.

Roberts CJ, Birkenmeier TM, McQuillan JJ, Akiyama SK, Yamada SS, Chen W-T, Yamada KM, McDonald JA. Transforming growth factor β stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts, J Biol Chem 1988;263:4586-92.

Mueller SC, Hasegawa T, Yamada SS, Yamada KM, and Chen W-T. Transmembrane orientation of the fibronectin receptor complex (integrin) demonstrated by a combination of immunocytochemical approaches, J Histochem Cytochem 1988;36:297-306.

Duband J-L, Dufour S, Yamada KM, Thiery JP. The migratory behavior of avian embryonic cells does not require phosphorylation of the fibronectin-receptor complex, FEBS Lett 1988;230:181-5.

Darribere T, Yamada KM, Johnson KE, Boucaut J-C. The 140-kDa fibronectin receptor complex is required for mesodermal cell adhesion during gastrulation in the amphibian Pleurodeles waltlii, Dev Biol 1988;126:182-94.

Piotrowicz RS, Orchekowski RP, Nugent DJ, Yamada KM, Kunicki TJ. Glycoprotein Ic-IIa functions as an activation-independent fibronectin receptor on human platelets, J Cell Biol 1988;106:1359-64.

Saga S, Nagata K, Chen W-T, Yamada KM. pH-dependent function, purification and intracellular location of a major collagen-binding glycoprotein,J Cell Biol 1987;105:517-29.

Nagata K, Saga S, Yamada KM. Characterization of a novel transformation-sensitive heat-shock protein (hsp47) that binds to collagen, Biochem Biophys Res Commun 1988; in press.

Nagata K, Hirayoshi K, Obara M, Saga S, Yamada KM. Biosynthesis of a novel transformation-sensitive heat-shock protein that binds to collagen: Regulation by mRNA levels and in vitro synthesis of a functional precursor, J Biol Chem 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08719-08 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

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)*

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.5	3.5	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 primary focus of work in the Molecular Genetics Section continues to be on growth regulation in mammalian cells. The investigation of growth inhibitory activity detected by DNA-mediated gene transfer is emphasized within this framework. Over the past year two techniques, magnetic affinity cell sorting (MACS) and fluorescence activated cell sorting (FACS) have been used in conjunction with gene transfer to measure transient growth regulation mediated by exogenous DNA sequences. Several results of interest have been obtained: i) at the polypeptide level, development of an efficient transfection protocol for human embryo fibroblasts has led to initiation of a project in which cDNA libraries are screened for genes involved in senescence and tumor suppression, ii) at the RNA level, evidence has been obtained for growth regulation, both positive and negative, by RNA polymerase III-encoded transcripts; mutational studies to derive structure/function relationships for 7SL RNA- and Alu-mediated growth control are in progress, iii) at the DNA level, cytotoxicity has been found to follow introduction of retroviral, e.g., HIV, long terminal repeat sequences; this finding may have implications for mechanisms of viral pathogenicity.

Other Professional Personnel:

M. Fordis	Expert	LMB, NCI
T. Howard	Guest Researcher	LMB, NCI
K. Sakamoto	Visiting Fellow	LMB, NCI
W. Holter	Visiting Fellow	LMB, NCI
C. Corsico	Guest Researcher	LMB, NCI
R.B. Helmly		LMB, NCI
S. Goldstein	Visiting Associate/Guest Worker	LMB, NCI
M. Willingham	Chief, Ultrastructural Cytochemistry Section	LMB, NCI

Major Findings:

I. Vector development and gene regulation studies. This project has been completed and results published.

II. New methods to improve DNA-mediated stable transformation efficiencies.

A. Sequential DEAE-dextran transfection method. Studies by Dr. Wolfgang Holter led to development of agene transfer protocol that yields up to 70% positive HeLa cells by FACS assay of Tac antigen expression. The protocol involves a brief treatment of cells with DEAE dextran followed by removal of excess polycation and incubation for atleast 3 hrs with DNA in the presence of chloroquine. A glycerol shock is performed after removal of DNA. This protocol is not only several times more efficient than the standard DEAE dextran-DNA coprecipitation procedure, but is also substantially less toxic for HeLa cells than the standard procedure.

B. Synergistic transfection enhancement by sodium butyrate and cell cycle synchronizing agents. A series of transfection experiments using human embryo fibroblasts indicated that none of the standard methods yielded satisfactory results. Electroporation at high voltages provided high transfection efficiency, but virtually abolished the capacity of treated cells to proliferate. To circumvent this problem, Dr. Goldstein investigated cell cycle synchronization and sodium butyrate treatment, two methods which had been previously reported to enhance cellular competence for the uptake and/or expression of foreign DNA. Surprisingly, he found that these two methods act synergistically to increase transfection efficiency from ≈4% to as high as 30%; most significantly, his results were obtained with electroporation conditions that did not seriously impair cellular replication capacity. Recent experiments suggest that the combination of synchronization and butyrate treatment is effective with other cell types and transfection methods.

III. Application of gene transfer methods to detection of genes that regulate cell growth.

A. Development of transfection/FACS assay: Detailed information concerning the replication of cells can best be obtained by FACS analysis. To this end, a protocol has been developed in which BrdUrd-labeled, pRSV-IL2R transfected cells are stained both for DNA synthesis (fluorescein-conjugated anti-BrdUrd) and surface marker expression (biotinylated anti-Tac + phycoerythrin-conjugated

streptavidin). Stained cells are analyzed on a Becton-Dickinson FACScan that employs a single argon laser. This assay has been verified using a known growth inhibitory gene, human β-interferon. As expected, HeLa cells transfected with the β-interferon gene exhibit decreased DNA synthesis; unexpectedly, they also exhibit a lengthening of S phase relative to other parts of the cell cycle. An additional set of experiments with this system has revealed that FACS-based estimation of the number of surface marker molecules (obtained by calibration against a fluorescence standard) agrees well with standard Scatchard plot analysis.

B. Inhibition of HeLa cell replication: This project has continued as the central focus of research in the the MGS. As noted at the conclusion of the 1987 annual report, we are investigating the possibility that RNA polymerase III (pol III)-encoded cellular RNAs are involved in regulating mammalian cell growth.

Over the past year, the majority of experiments designed to study the role of small RNAs in growth control have concentrated on the 7SL RNA/Alu gene family. This family was selected for study for a number of reasons: i) increased levels of 7SL RNA were detected in human embryo fibroblast (HEF) DNA-transfected HeLa cell lines that had been selected for slowed growth, ii) the partial homology between 7SL RNA and Alu sequences provided a possible explanation for the apparent interspersed repetitive nature of inhibitory sequences observed our earlier experiments, and iii) 7SL RNA appeared to have interesting potential as a growth regulatory molecule based on its known function as a component of the signal recognition particle (SRP). Since the SRP is required for translocation of nascent peptides into the endoplasmic reticulum, it is essential for transport of both proteins that are destined for the cell surface and proteins that are to be secreted. The potential link to cellular display of cell surface proteins includes receptors for growth factors, both positive and negative; likewise, the link to secreted proteins includes extracellular growth regulatory proteins.

Both MACS- and FACS-based protocols have been used to test the potential role of 7SL RNA in regulating HeLa cell growth. In the MACS assay, we found that transfection of HeLa cells with a plasmid carrying the 7SL RNA gene decreased ^{3}H-thymidine incorporation into IL2 receptor positive cells by several fold relative to controls. The 7SL RNA gene decreased expression of cotransfected pRSV-IL2R and pRSVcat plasmids by a comparable amount, suggesting that overall cellular growth and/or protein synthesis were suppressed. In the FACS/BrdUrd assay, decreased BrdUrd incorporation was detected in two experiments, but further studies are needed to rigorously exclude potential assay artifacts.

To gain more insight into 7SL RNA-mediated HeLa cell growth suppression, structure-function studies have been initiated. The central S region of 7SL RNA is known to suffice for signal sequence recognition and ER docking by the SRP, whereas the Alu domain is thought to function in elongation arrest by the SRP. Deletion of the central S region of the gene left growth inhibitory activity largely intact. This suggests that growth regulatory activity resides primarily in the Alu region; moreover, since the S region-deleted clone should be

completely defective in signal sequence binding, the remaining Alu region in that construct is unlikely to inhibit growth by abnormal elongation arrest. It follows that the Alu domain of 7SL RNA may possess function(s) unrelated to SRP activity. Additional support for Alu region function comes from an experiment in which mutation of the "ori" site in the 5' Alu domain substantially reduced growth inhibitory activity.

More direct evidence for a potential role for pol III-transcribed Alu interspersed repetitive sequences in HeLa cell growth control has been obtained by testing a variety of Alu-containing plasmids in the MACS assay. Two of the tested Alu clones were derived from low Cot reannealing of genomic DNA (BLUR2 and BLUR8), two clones were derived from specific genomic loci (3α1 and AMA-L), and one clone was constructed by chemical DNA synthesis (AluK). The latter is at least 99% homologous to the human Alu consensus sequence. Each of these clones was active, demonstrating that the potential to suppress HeLa cell growth is a general property of Alu SINEs. As in the case of the 7SL RNA gene, a mutation in the ori site near the 5' end of the BLUR8 Alu clone abolished most growth inhibitory activity. Further mutational studies will be required to map the domains that are essential for activity and to determine whether primary sequence or secondary structure (or both) are involved.

In parallel with studies on the 7SL RNA/Alu family, growth regulatory properties of other DNA sequences and genes have been examined in both the MACS and FACS assays. HeLa cell growth inhibition/cytotoxicity has been observed within several hours after transfection of certain sequences by the standard DEAE dextran-DNA coprecipitation procedure. Long terminal repeats (LTRs) from retroviruses, including HIV, and from the transposon-like human element THE-1A are particularly active in this respect. We postulate that such growth inhibition/cytotoxicity occurs at the DNA level, with transfected exogenous DNA sequences titrating essential cellular growth or transcription factors. This observation may be relevant to the mechanism of viral pathogenicity of retroviruses that replicate to high copy number, e.g., spleen necrosis virus and perhaps HIV. We are also interested in finding DNA sequences that stimulate cell growth at the DNA level. Such sequences could provide a "handle" to identify intracellular growth inhibitory proteins.

C. Regulation of growth in cell types other than HeLa. To broaden the base of our studies on growth regulation, we have initiated transfection studies using monkey kidney CV-1 cells, an immortalized mouse fibroblast line (BALB/3T3), and non-immortalized human embryo fibroblasts (HEF). It proved necessary to modify transfection and MACS techniques to obtain optimal results with each cell type.

MACS experiments with CV-1 cells indicate that 7SL RNA may be bifunctional with respect to growth, since increased ^{3}H-thymidine incorporation in the sorted population is observed following calcium phosphate-mediated transfection of those cells with the wild type 7SL RNA gene. This observation provides support for the idea that HeLa cell growth suppression by 7SL RNA is physiological (since other well accepted growth factors are bifunctional); however, analysis by the FACS protocol will be required to confirm that growth stimulation rather than increased IL-2 receptor display accounts for the enhancement in ^{3}H-thymidine incorporation.

MACS experiments with HEF so far have focused almost exclusively on screening of cDNA libraries for genes involved in senescence. Although no unambiguously inhibitory pools or clones have been identified, the establishment of a technique to study growth regulation in normal HEF represents a very significant advance in our long term project to understand the molecular mechanisms underlying tumor suppression and senescence.

Publications:

Padmanabhan R, Howard TH, Howard BH. Specific growth inhibitory sequences in genomic DNA from quiescent human embryo fibroblasts, Mol Cell Biol 1987;7: 1894-9.

Fleischmann R, McCormick M, Howard BH. Preparation of a genomic cosmid library, Methods Enzymol 1987;151:405-16.

Fordis CM, Howard, BH. Use of the CAT reporter gene for optimization of gene transfer into eucaryotic cells, Methods Enzymol 1987;151:382-97.

McCormick M, Gottesman ME, Gaitanaris GA, Howard BH. Cosmid vector systems for genomic DNA cloning, Methods Enzymol 1987;151:397-405.

Padmanabhan R, Howard TH, Howard BH. Inhibition of HeLa cell growth following transfection with genomic DNA from human embryo fibroblasts. In: Colburn NH, Moses HL, Stanbridge EJ, eds. Growth Factors, Tumor Promoters, and Cancer Genes. New York: Alan R Liss Inc, 1988;369-76.

Holter W, Majdic O, Stockinger H, Howard BH, Knapp W. Regulation of the CD2 alternate pathway of T cell activation by CD3, J Immunol 1988;140:1043-6. 1988.

Padmanabhan R, Corsico CD, Howard TH, Holter W, Fordis CM, Willingham M, Howard BH. Purification of transiently transfected cells by magnetic affinity cell sorting, Anal Biochem 1988;170:341-8.

Howard BH. A potential role for interspersed repetitive sequence elements in negative growth regulation. In: Wang E, Warner HR, eds. Growth control during cell aging. Boca Raton, FL: CRC Press Inc, 1988; in press.

Corsico CD. Howard BH. Chloramphenicol acetyltransferase as a reporter gene in mammalian gene transfer experiments. In: Pollard JW, Walker JM, eds. Methods in Molecular Biology, Vol 5. Clifton, NJ: Humana Press Inc, 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08750-08 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

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
	S. Garges	Microbiologist	LMB, NCI
Other:	J. Kim	Biotechnology Fellow	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Developmental Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	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.)*

Cyclic AMP binds to its receptor protein CRP, and induces a conformational change (allosteric transition) in the protein. Cyclic AMP·CRP complex, and not free CRP, binds at or near many promoters of E. coli in a sequence-specific way and either represses or activates transcription. We have isolated and characterized several classes of mutations in the crp gene, which identify the amino acids participating in the cyclic AMP-induced allosteric shift in the protein. On the basis of these results and the available three-dimensional structure of the protein, we proposed that cyclic AMP brings about: (1) a hinge reorientation to eject the DNA-binding F α-helices; (2) proper alignment between two subunits; (3) an adjustment between the position of the two domains. Extensive amino acid substitution at some of these positions further supports the idea that hinge movement involving interhelical polar-polar interactions and/or Van der Waals repulsion are an essential part of the activation of CRP. We have further shown, by site-directed mutagenesis, that a hydrogen bond formation between amino acids 171 and 63 participates in domain-domain alignment for CRP activation. Using a DNA bending vector developed in this laboratory, we have found that CRP binding induces DNA bending at all tested promoters with a CRP binding site whether the promoters are activable or repressible by CRP. The following two findings strongly support the idea that CRP activates RNA polymerase by a direct protein-protein contact and not by sending a signal through DNA: (1) We have found that substantially increasing the distance between the RNA polymerase binding site and the CRP binding site in lacP does not affect CRP activation of lacP. (2) Additionally, we have clearly demonstrated, using one of our CRP mutants, that CRP and RNA polymerase bind to DNA co-operatively.

GPO 914-918

Major Findings:

We are studying the cAMP receptor protein (CRP), a protein that binds to DNA and can activate or repress the transcription of many operons of Escherichia coli. Study of such a protein provides a model system for investigation of transcriptional activators and repressors, and provides information on protein-protein interaction and DNA-protein interactions.

We are studying three different aspects of CRP:

A. The cAMP-induced allosteric change of CRP.

CRP is inactive for site-specific DNA binding unless cAMP is bound. When cAMP binds, the protein undergoes a conformational change. We have previously isolated and sequenced several cAMP independent CRP mutants. They are located in the hinge connecting the DNA-binding and cAMP-binding domains. They substituted amino acids with a polar or a longer side chain (Gly141→SER and Ala144→Thr). On the basis of the three-dimensional structure and our results, we have proposed the following model. cAMP brings about a hinge reorientation to eject the DNA-binding F α-helices, proper alignment between the two subunits, and an adjustment between the position of the two domains. The following results obtained during the past year provided the evidence for the support of our model.

1. From extensive amino acid substitutions either at position 141 or 144, we have found that at position 141 polar amino acids allow cAMP independence, while at position 144 large amino acids permit cAMP independence. We believe that these two mutations bring about the similar structural change in CRP. At position 141 the interhelical polar-polar interaction would close the hinge region, eventually freeing the DNA-binding F α-helix. At position 144 the direct pushing against the F α-helix by large amino acids would set free the α-helix for specific DNA binding. Thus, the results show that the hinge movement is essential part of the activation of CRP.

2. We have further investigated the interhelical interactions in the hinge region, specifically between amino acids Gly141 and Asp138. By creating double mutations at these two positions we have found that polar amino acids at both positions were needed for cAMP independent phenotype. Thus, these results also show that the hinge movement is required for the activation of CRP.

3. We have investigated the role of amino acids Glu171. Glutamic acid at position 171 in the wild type protein has been suggested to form a hydrogen bond with a tyrosine at position 63, thus creating one of the major contacts between the cAMP-binding domain and the DNA-binding domain. Upon substitution of Ser, Gln, Tyr or Leu at position 171, the proteins became inactive. Thus, these results suggest the importance of a domain-domain interaction for the activation of CRP.

4. We are searching for more sites at which cAMP acts by continuing our previous studies to isolate crp mutants that are more cAMP-independent than our current mutants.

B. CRP•cAMP-induced DNA bending.

It is not known whether the DNA-bending ability of CRP is related to its transcription activation or repression capabilities. To study this aspect of CRP, we have taken two different approaches.

1. Using a DNA bending vector developed in this laboratory , we have tested different CRP binding sites for the ability of CRP to bend the DNA. We have found that certain sites bend more than others when CRP is bound. We have found that at sites where CRP is known to repress (inhibit) transcription, there is still DNA bending.

2. We have initiated a selection for crp mutants that can bind to DNA but cannot bend the DNA. We have several potential candidates (CRPs that can bind to DNA but do not activate) but they have not yet been characterized for their DNA-bending abilities.

C. The mechanism by which CRP activates transcription.

How CRP activates transcription is unknown, but current models suggest either a change in conformation of the promoter transmitted for the DNA where CRP is bound or a direct interaction with RNA polymerase. We have data to support the latter model, and we are continuing to investigate this important aspect of CRP.

1. We have changed the distance between the lac promoter and its cognate CRP binding site. Preliminary results are that insertion of 117 base pairs does not appear to inhibit significantly CRP activation of lacP. This supports the CRP-RNA polymerase contact model (the proteins contacting by DNA looping) because it is unlikely that a structural change in the DNA more than 130 base pairs away would be transmitted to the promoter.

2. Using one of our previously-isolated cAMP-independent crp mutants, we have found that the CRP will not bind to DNA (in DNase footprinting experiments) unless RNA polymerase is first bound to the lac promoter. This supports a model with RNA polymerase and CRP interacting since it appears that RNA polymerase will help CRP to bind to DNA.

3. We have initiated a search for crp mutants that can activate transcription although no CRP binding site is present. Although we had to go through two rounds of mutagenesis (suggesting such mutants are not easily obtained), we have found crp mutants that are potentially DNA-independent. They are currently being characterized.

Publications:

Garges S, Adhya S. Cyclic AMP-induced conformational change of cyclic AMP receptor protein (CRP): Intragenic suppressors of cyclic AMP independent CRP mutations, J Bacteriol 1988;170:1417-22.

Ren YL, Garges S, Adhya S, Krakow J. Co-operative DNA binding of heterologous proteins: Evidence for contact between the cyclic AMP receptor protein and RNA polymerase, Proc Natl Acad Sci USA 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08751-08 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

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:	R. Haber	Microbiologist	LMB, NCI
	J. Tokeson	Student Volunteer	LMB, NCI
	N. Mandal	Visiting Scientist	LMB, NCI
	C. Zwieb	Visiting Scientist	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Developmental Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

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.)*

Gal repressor protein negatively regulates the expression of the gal operon of E. coli. Repression requires binding of Gal repressor to two operator sites, O_E and O_I, which are separated from each other by 114 bp and flank two promoters of the operon. Repressor binding to O_E and O_I is co-operative. However, occupation of the operators by repressor is not sufficient to achieve normal repression and requires an interaction between the two operator-bound repressor molecules. Replacement of one of the gal operators by a lac operator causes derepression of the gal operon in the presence of both Gal and Lac repressors, whereas, substitution of both operators by lac operator sequences re-establishes repression. We have proposed that the two repressor molecules bound to the spatially separated operators associate giving rise to a higher order structure containing a DNA loop with-bound repressor, CRP and RNA polymerase. The DNA in this complex is altered and inactive for transcription. The following results are consistent with our model. (1) By analyzing repressor mutants which bind to DNA but do not associate and, thereby, fail to bring about repression, we have demonstrated that repressor-repressor contact is needed for repression. (2) Wehave shown that repressor binding induces structural changes in DNA including DNA bending and change in circular dichrosim spectra.

Major Findings:

1. We have investigated the functional relationship between the operators, O_E and O_I, of the gal operon of E. coli. By site-directed mutagenesis, we have constructed strains in which one or both gal operators were converted to lac operator sequences. We have found that strains with homologous operators, either two gal operators or two lac operators, can be fully repressed by the cognate repressor protein, but strains with heterologous operators, one lac and one gal operator are not fully repressed even when both Gal and Lac repressors are present in excess. Thus, mere occupation of the two operators by repressor is not sufficient for repression; the two operator-bound repressor molecules must function interdependently. Moreover, our results also show that Lac repressor can function like Gal repressor to achieve negative regulation from distal sites.

2. We have measured the repressor binding affinities in vivo of the variously converted operators described above by a method previously developed by us.Our results conclusively show that the two operators, O_E and O_I, bind to repressor in a co-operative fashion. The affinity of repressor to O_E and O_I present in cis is higher than the sum of the affinities to O_E and O_I.

3. Using the same in vivo binding assay method, we have shown that the location of the operator in the operon affects its ability to bind repressor (position effect). An identical operator DNA sequence will bind repressor protein more efficiently when located at the external site than when located at the internal site.

4. We have confirmed the in vivo repressor binding patterns of the variously converted operators using purified repressors and ^{32}P-labeled DNA by the millipore filter binding assays.

5. We have characterized a mutant repressor which remains as a dimer and is defective in tetramerization. The mutation is due to a single frameshift deletion of a GC base pair in the carboxy end of the repressor gene. It results in the truncation of the monomer by 10 amino acids. This mutant repressor binds to the operators but fails to repress the operon. Further characterization of this mutant is in progress.

6. We have shown that Gal repressor binding to 22 bp O_E and O_I DNA increases the circular dichroism spectrum of the DNA in the 280-300 nm range. This is consistent with the idea that repressor binding induces conformational changes in DNA. We have also shown that mutation of the central (8 and rotational 8') GC base pairs of the 16 bp O_E sequence to AT base pairs abolishes the repressor-induced increase in CD spectra in DNA, whereas mutation of the GC base pairs at the perimeters (1 and rotational 1') does not affect the increase in CD spectra. These results suggest that the central GC base pairs participate in the repressor induced increase in the CD spectra of DNA.

7. we have previously proposed a model by which Gal repressor acts. Two Gal repressor molecules binding to spatially separated O_E and O_I interact to give rise to a higher order structure in which the promoter DNA segment is present as

a loop and bound to RNA polymerase and CRP. The promoter DNA and/or RNA polymerase present in this higher order structure has altered conformation and is inactive for transcription. The results reported above support different aspects of this model.

Publications:

Adhya S. The galactose operon. In: Neidhardt FC, ed. Escherichia coli and Salmonella typhimurium. Washington: American Society of Microbiology, 1987;1503-12.

Adhya S, Majumdar A. Negative control of the gal operon. In: Reznikoff W, Burgess R, Dahlberg J, Gross C, Record T, Wickens M, ed. RNA polymerase and regulation of transcription. New York: Elsevier Science Publishing Co 1988; 129-135.

Majumdar A, Adhya S. Probing the structure of Gal operator-repressor complexes: DNase and DMS protection studies, J Biol Chem 1987;262:13258-62.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08752-08 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

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

PI:	S.-y. Cheng	Research Chemist	LMB, NCI
Other:	C. Parkinson	Chemist	LMB, NCI
	T. Fukuda	Visiting Fellow	LMB, NCI
	H. Kato	Visiting Fellow	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

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.)*

I. High level expression of the endoplasmic reticulum-associated thyroid hormone binding protein (p55). A full-length p55 cDNA was inserted into a Harvey murine sarcoma virus-derived vector (pHTBr). pHTBr was transfected into NIH 3T3 cells using pRSVneo and pHaMDR1 as the selectable markers. Using indirect immunofluorescence, nine stable cell lines expressing p55 were identified. Among the nine, two cell lines were expressing p55 as high as that in parental cells. Furthermore, the molecular size, immunoreactivity, subcellular localization and the reactivity of the expressed p55 with an affinity analog of 3,3',5-triiodo-L-thyronine (T_3), N-bromoacetyl-T_3 are indistinguishable from those of the endogenous p55. These results indicated the production of stable cell lines expressing heterologous p55. These stable cell lines could be used as a tool to further study the function of p55.

II. Characterization of a cytosolic thyroid hormone binding protein (p58) using monoclonal antibodies. A cellular hormone binding protein (p58) was purified to homogeneity. Two hybridomas, J11 and J12, secreting monoclonal antibodies to p58 were isolated. Using these antibodies, p58 was found to be not post-translationally modified by glycosylation, sulfation or phosphorylation. It has a cellular degradation rate, $t_{1/2}$ = 2.1 hour. Immunocytochemical studies indicate that p58 is located in the non-membranous cytoplasm (cytosol). Its role in the intracellular transport of T_3 is being investigated.

III. Antipeptide antibodies to the T_3 nuclear receptor. Recent studies have shown that c-erbA protein is the T_3 nuclear receptor. To study its tissue distribution and subcellular localization, antipeptide antibodies to the human placenta c-erbA (hc-erbA-β) protein were prepared. Two antibodies, NP-98 and CP-91, specifically reacted with the hc-erbA-β protein synthesized in vitro. These antibodies also specifically immunoabsorb the T_3 binding activity of the T_3 nuclear receptors isolated from placenta tissue and cultured cells. These antibodies are a valuable tool in studying the thyroid hormone action.

Major Findings:

I. High level expression of the endoplasmic reticulum-associated thyroid hormone binding protein (p58).

A cDNA encoding the endoplasmic reticulum-associated thyroid hormone bindingprotein was isolated. Sequence analysis indicated that p55 is homologous to the protein disulfide isomerase and the ß-subunit of prolyl-4-hydroxylase. Protein disulfide isomerase has been shown to catalyze the rearrangement of disulfide bonds of various proteins in vitro. Its in vivo enzymatic activity, however, has not been demonstrated. To study the function of p55 and its roles in T_3 action, a simple efficient system for a high level expression of p55 in eukaryotic cells was developed.

A full-length p55 cDNA was inserted into a Harvey murine sarcoma virus-derived vector (pHTBr). pHTBr was co-transfected into mouse NIH 3T3 cells with pRSVneo or pHaMDR1, an expression vector for the dominant, selectable human multidrug-resistance gene. Nine cell lines expressing p55 were isolated; two lines G2 and C2 expressed more than 10^6 molecules per cell. The expressed thyroid hormone binding protein has a molecular weight of 55,000 and is recognized by the human specific anti-p55 monoclonal antibody. Similar to the endogenous p55, the expressed p55 is localized on the endoplasmic reticulum and nuclear envelope. Moreover, p55 was affinity labeled by N-bromoacetyl-[^{125}I]T_3, an affinity analog of T_3. The labeling was inhibited in the presence of unlabeled T_3. Taken together, these results indicated the production of stable cell lines expressing heterologous p55. These cell lines provided a tool for studying effects of high level expression of p55 on cellular functions.

II. Characterization of a cytosolic thyroid hormone binding protein (p58) using monoclonal antibodies.

A cellular thyroid hormone binding protein which differs from p55 (see above) was purified to homogeneity. The purified p58 was examined for its binding activity to the thyroid hormones. Analysis of binding data indicate that p58 binds to 3,3',5-triiodo-L-thyronine (T_3) with a Kd of 24.3 ± 0.3 nM and N = 0.71. p58 binds to L-thyroxine similarly as T_3. However, D-T_3 and reverse-T_3 bind to p58 with an affinity 4- to 20-fold less that T_3, respectively. These data indicate that the purified p58 retains its binding activity and specificity. The purified p58 was used as immunogen to prepare monoclonal antibodies. Two hybridomas, J11 and J12, secreting monoclonal antibodies to p58 were isolated; both antibodies belong to the IgG_{1k} subclass. J12 recognizes p58 from human, monkey, dog, hamster and rat, but not mouse. J11 exhibits a similar species specificity except that it does not react with p58 from hamster.

Using these antibodies, p58 was further characterized. p58 was found to be present in five human cultured cell lines examined, the highest abundance was found in KB cells, the least abundance in HepG2 cells. p58 was not a protein which was post-translationally modified by glycosylation, sulfation or phosphorylation. Its cellular degradation has two components, one is fast decaying (half-life of 2.1 hours) and the other is a stable pool. Immuno-fluorescence and electronmicroscopic studies showed that it is exclusively present in the non-membranous cytoplasm.

Peptide mapping of p58 and immunologic studies indicate that p58 is not structurally related to c-erbA protein. p58 could serve as an intracellular T_3 transporter or as a buffer to maintain intracellular concentration of T_3. Its functions are currently being evaluated.

III. Antipeptide antibodies to the T_3 nuclear receptors.

Recent studies have shown the c-erbA protein is the thyroid hormone nuclear receptor. To study its tissue distribution and subcellular localization, antipeptide antibodies to the human placenta c-erbA (hc-erbA-β) protein were prepared. Peptides corresponding to the deduced amino acid residues 15-29 of the amino terminal region and 445-456 of the carboxyl terminal of hc-erbA-β were synthesized and used to produce site-specific rabbit polyclonal antipeptide sera. Antibodies to the carboxyl terminal peptide 445-456 (CP-91) immunoprecipitated the in vitro translation products (MWt: 55K, 52K, and 35K) of hc-erbA-β; whereas, antibodies to the amino terminal peptide 15-29 (NP-98) immunopre-cipitated only 55k and 52K proteins. Immunoprecipitation of the above proteins was inhibited by the presence of excess corresponding peptide. These results indicated that CP-91 and NP-98 are specific against the hc-erbA-β proteins.

To evaluate whether NP-98 and CP-91 recognize the T_3 nuclear receptor isolated from the nuclei of placenta and cultured cells, nuclear extracts were prepared. Preincubation of CP-91 with the hc-erbA-β synthesized in vitro, nuclear extracts of human placenta or A431 cells, reduced the T_3 binding by 40%, 30%, and 45%, respectively. These results indicated that CP-91 recognizes the T_3 nuclear receptor. Thus these results provided the direct structural evidence for the first time to show that the c-erbA protein is the T_3 nuclear receptor.

To evaluate whether c-erbA protein is present in human cultured cells, human A431 cells were metabolically labeled with [^{35}S]methionine and the cellular extracts were immunoprecipitated with NP-98 or CP-91. A protein with a molecular weight of 58,000 (Cp58) was immunoprecipitated by either NP-98 or CP-91. The immunoprecipitation was blocked by the presence of the corresponding peptide. Peptide mapping by V8 digestion and the cyanogen bromide (CNBr) cleavage showed that the Cp58 molecules immunoprecipitated by NP-98 or CP-91 are identical. No specific protein was detectable by immunoprecipitation of the nuclear extracts by either antibody. This could be due to the possibility that the method is not sensitive enough to detect trace amounts of the T_3 nuclear receptor. Comparison of the peptide maps obtained by V8 digestion and CNBr cleavage between the hc-erbA-β synthesized in vitro and the p58 showed the existence of several common fragments, indicating some similarity in the protein sequences. Indirect immunofluorescence using NP-98 and CP-91 indicated that the Cp58 is present exclusively in the cytoplasm. These results raised the possibility that a precursor of hc-erbA-β may be present in the cytosol. The functional significance of the hc-erbA-β related cytosolic p58 remains to be established.

Publications:

Kitagawa S, Obata T, Willingham MC, Cheng S-y. Thyroid hormone action: Induction of morphological changes and stimulation of cell growth in rat pituitary tumor GH_3 cells, Endocrinology 1987;120:2591-6.

Cheng S-y, Gong QH, Parkison C, Robinson EA, Appella E, Merlino GT, Pastan I.The nucleotide sequence of a human cellular thyroid hormone binding protein present in endoplasmic reticulum, J Biol Chem 1987;262:11221-7.

Willingham MC, Rutherford AV, Cheng S-y. Immunohistochemical localization of a thyroid hormone binding protein, J Histochem Cytochem 1987;35:1043-6.

Popescu N, Cheng S-y, Pastan I. Chromosomal localization of the gene for a human thyroid hormone binding protein, Am J Human Genet 1988;42:560-4.

Obata T, Kitagawa S, Gong QH, Pastan I, Cheng S-y. Thyroid hormone down regulates p55, a thyroid hormone binding protein that is homologous to protein disulfide isomerase and the β-subunit of prolyl 4-hyroxylase, J Biol Chem 1988; 263:782-5.

Obata T, Fukuda T, Cheng S-y. Antibodies against the human cellular 3,3', 5-triiodo-L-thyronine-binding protein (p58), FEB 1988;230:9-12.

Obata T, Cheng S-y. Strip-comb dot immunobinding: A rapid, easy and sensitive method to screen monoclonal antibodies, BioTechniques 1988;6:299-302.

Gong Q-h, Fukuda T, Parkison C, Cheng S-y. Nucleotide sequence of a full-length cDNA clone encoding a mouse cellular thyroid hormone binding protein (p55) that is homologous to protein disulfide isomerase and the β-subunit of prolyl-4-hydroxylase, Nucleic Acids Res 1988;16:1203.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08753-06 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Immunotoxin and Oncotoxin Therapy of Cancer Cells

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

PI:	I. Pastan	Chief, Laboratory of Molecular Biology	NCI
	M.C. Willingham	Chief, UCS	LMB, NCI
	D. FitzGerald	Microbiologist, UCS	LMB, NCI

COOPERATING UNITS *(if any)*

Metabolism Branch, DCBD, NCI
Medicine Branch, DCT, NCI
Merck Corporation
Hoffmann-La Roche, Inc.

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
11.0	8.0	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.)*

Pseudomonas exotoxin (PE) or genetically modified forms of PE have been attached to monoclonal antibodies (mAbs) or growth factors to create cell-specific cytotoxic agents. Native PE attached to mAb OVB3 is cytotoxic to ovarian cancer cells, and to ovarian cancer in mice, and is being currently tested in women with ovarian cancer. Mutant PE molecules in which domain I has been replaced by TGFα or IL2 have been created by gene fusion and the chimeric proteins, TGFα-PE40 and IL2-PE40, produced in E. coli. TGFα-PE40 is very cytotoxic to cells with EGF receptors and IL2-PE40 is cytotoxic to cells with IL2 receptors. Such molecules may be useful in the elimination of tumor cells or other cells responsible for several human diseases.

GPO 914-918

Other Professional Personnel:

S. Adhya	Chief, Developmental Genetics Section	LMB, NCI
V. K. Chaudhary	Visiting Associate	LMB, NCI
H. Lorberboum-Galski	Visiting Fellow	LMB, NCI
T. Idziorek	Visiting Fellow	LMB, NCI
Y. Jinno	Visiting Fellow	LMB, NCI
Y.-h. Xu	Visiting Fellow	LMB, NCI
M. Ogata	Visiting Fellow	LMB, NCI
T. Kondo	Visiting Fellow	LMB, NCI
T. Waldmann	Metabolism Branch	NCI
M. Bookman	Medicine Branch, DCT	NCI

Major Findings:

Pseudomonas exotoxin (PE) is a single chain bacterial toxin. Its mechanism of action is to inhibit mammalian cell protein synthesis by ADP ribosylating elongation factor 2 (EF-2). The toxin is composed of three structural domains: domain I is for cell recognition, domain II mediates translocation of the toxin to the cell cytoplasm and domain III ADP ribosylates EF-2.

1. Various forms of PE have been cloned and expressed in E. coli using a T7 promoter based expression system. One form is PE40 that contains the translocating and ADP-ribosylating domains of PE (domains II and III). A closely related form of this protein is LysPE40 which has an extra lysine placed near the amino terminus to facilitate coupling to antibodies. LysPE40 has been purified and chemically cross-linked to various monoclonal antibodies. The advantage of using LysPE40 in place of native PE is to avoid the liver toxicity associated with administering immunotoxins made with native PE. The injection of 2 - 4 μg of a PE-immunotoxin is lethal for a 20 gram mouse but up to 100 μg of a LysPE40 immunotoxin can be given without toxicity.

2. When LysPE40 was coupled to antibodies to the transferrin receptor (HB21) or to the IL2 receptor (antiTac) active cell-killing immunotoxins were made which had similar ID_{50}s to the corresponding PE-immunotoxins. However, OVB3-LysPE40 had no cell-killing activity. Apparently, the OVB3 antibody binds to ovarian cancer cells but does not mediate internalization. A portion of domain I of PE is probably responsible for the internalization of OVB3-PE. A plasmid encoding domain I has been expressed in E. coli and the protein (domain I) produced purified and used to compete with OVB3-PE. Domain I blocked the activity of OVB3-PE supporting the notion that the killing activity of OVB3-PE requires that domain I bind to the target cell.

3. Domain I contains 12 lysine residues. All of these have been changed to glutamate residues by site directed mutagenesis and lysine 57 has been shown to be important for the binding of PE to its receptor. Replacing lysine 57 with glutamate reduced the cell-killing activity of PE for tissue culture cells by 100-fold. To investigate the role of lysine 57 in OVB3-PE mediated cell-killing, PE^{glu57} was expressed, purified, coupled to OVB3 and assayed for cell-killing activity. OVB3-PE^{glu57} had no cell-killing activity. Thus, lysine 57 was needed both for PE-binding and PE-mediated internalization of the OVB3 immunotoxin.

4. PE^{glu57} was also tested for animal toxicity and, surprisingly, had very similar toxicity to native PE. By making a series of deletions in domain I, we located a region responsible for liver toxicity and found it was distant from lysine 57. PE40 (amino acids 253-613) had little toxicity for mice, whereas, PE43 (amino acids 223-613) had close to full liver toxicity. Thus, domain I of PE has two cell recognition regions: lysine 57 for cell-killing of tissue culture cells; and a region bounded by amino acids 223 and 253, which somehow produces liver toxicity.

5. The secretion by E. coli of various deletion derivatives of PE was studied in some detail. Full-length PE accumulated inside the cell, but deletion of domain I resulted in the secretion of domain II and III (PE40) into the periplasm. Further deletion of portions of domain II caused some of the PE protein (i.e., part of domain II and domain III) to be secreted into the culture medium. Domain II alone was found mostly in the periplasm while domain III alone was present inside the cell. Plasmids were also created in which an OmpA signal sequence was placed 5' to the PE gene. Addition of this signal sequence resulted in the secretion of PE into the periplasm. Addition of an OmpA signal sequence to PE40 resulted in PE40 appearing in the culture medium. N-terminal sequence analysis showed that the signal sequence was correctly processed. These results indicate that a part of domain II has an important role in the secretion of PE by E. coli.

6. A cDNA encoding transforming growth factor type alpha (TGFα) was fused to the 5' end of PE genes in which all (PE40) or most (PE43) of domain I was deleted. The chimeric proteins are termed TGFα-PE40 and TGFα-PE43. Without a signal sequence present, large amounts of TGFα-PE40 and TGFαPE43 were secreted into the periplasm. When an OmpA signal sequence was added, considerable amounts were secreted into the medium. TGFα-PE40 and TGFα-PE43 were purified and found to be extremely cytotoxic to cells bearing EGF receptors. On A431 cells, the ID_{50}s were 0.6 ng/ml and 0.05 ng/ml. TGFα-PE40 and TGFαPE43 were toxic to mice with an $LD_{50} < 30$ μg for TGFαPE40 and 5 μg for TGFαPE43.

7. A cDNA clone for human interleukin 2 (IL2) was also fused to the 5' end of PE40 and the chimeric protein, IL2-PE40, was produced in E. coli. The fusion protein was extracted from inclusion bodies using GuHCl and purified by receptor affinity chromatography yielding a highly purified active monomeric form of the protein. IL2-PE40 is extremely toxic to IL2-receptor positive cells but has no measurable cytotoxic effect on cells lacking the IL2-receptor. IL2-PE40 has low nonspecific toxicity in mice (LD_{50} ~50 μg) and rats. The cytotoxicity of IL2-PE40 was tested on cells expressing either p55, p75, or both IL2 receptor subunits. IL2-PE40 inhibited protein synthesis on cell lines expressing either p55 or p75.

To investigate the immunosuppresive effect of IL2-PE40, we examined whether activated T lymphocytes expressing IL2-receptors were sensitive to IL2-PE40. We found that IL2-PE40 was extremely cytotoxic to concanavalin A-activated T lymphocytes. Moreover, IL2-PE40 was cytotoxic to antigen-activated T lymphocytes in a mixed lymphocyte culture, which is an in vitro model system for an anti-allo immune response.

8. To create antibody-toxin fusion proteins, the heavy chain gene encoding the rearranged variable region of mAb OVB3 was cloned. Plasmids were created to express PE40 fused to the heavy chain of an antibody. First, using a gene encoding a rearranged heavy chain variable region of an antibody that recognizes a hapten (Nitro-phenyl), the plasmid, pSVVnp-PE40, was constructed. This plasmid carries a rearranged heavy chain gene encoding the variable region, CH1, hinge region and the first five amino acids of CH2 fused to the PE40 gene and GPT as selection marker. pSVVnp-PE40 was transfected into cell line J558L which produces light chains and transfectants selected with mycophenolic acid. Clones expressing the fusion protein were identified by analyzing the supernatants in an ELISA which detects antigen (Nitro-phenyl) binding and reactivity to antibodies to PE. Several clones were isolated that express and secrete antibody-PE fusion proteins, but the fusion proteins were smaller in size than expected.

9. A Phase I clinical study using OVB3-PE to treat patients with refractory ovarian cancer has been begun in the Medicine Branch, DCT, NCI. Four patients have been treated at a low dose level (1 µg/kilo) without serious side effects.

Publications:

Pirker R, FitzGerald D, Willingham MC, Pastan I. Immunotoxins and endocytosis. In: Pick E, eds. Lymphokines Vol 14. Orlando Fl: Academic Press, 1987;361-382.

Willingham MC, FitzGerald DJ, Pastan I. Pseudomonas exotoxin coupled to a monoclonal antibody against ovarian cacner inhibits the growth of human ovarina cancer cells in a mouse model, Proc Natl Acad Sci USA 1987;84:2474-8.

Seth P, Pastan I, Willingham MC. Adenovirus-dependent changes in cell membrane permeability: Role of Na^+, K^+ ATPase, J Virol 1987;61:883-8.

Cryz SJ Jr, Furer E, Sadoff JC, Germomier R, Pastan I, Willingham MC, FitzGerald DJP. The use of Pseudomonas Aeruginosa toxin A in the construction of conjugate vaccines and immunotoxins, Rev Infect Dis 1987;9:S644-9.

Lorberboum-Galski H, FitzGerald D, Chaudhary V, Adhya S, Pastan I. Cytotoxic activity of an interleukin 2-pseudomonas exotoxin chimeric protien produced in E. coli, Proc Natl Acad Sci USA 1988;85:1922-6.

Chaudhary VK, Xu Y-h, FitzGerald D, Adhya S, Pastan I. Role of domain II of pseudomonas exotoxin in the secretion of proteins into the periplasm and medium by E. coli, Proc Natl Acad Sci USA

Pirker R, FitzGerald DJP, Willingham MC, Pastan I. Enhancement of the activity of immunotoxins made with either ricin A chain or Pseudomonas exotoxin, Cancer Res 1988; in press.

Gallo M, Chaudhary V, FitzGerald DJP, Willingham MC, Pastan I. Cloning and expression of the heavy chain variable region of antibody OVB3 that reacts with human ovarian cancer, J Immunol 1988; in press.

FitzGerald DJ, Willingham MC, Pastan I. Pseudomonas exotoxin--Immunotoxins. In: Frankel A, ed. Immunotoxins. Martin Nijoff BV, 1988; in press.

FitzGerald D, Pastan I. Toxin conjugates: Interactions with mammalian cells. In: Saelinger CB, ed. Trafficking of bacterial toxins. CRC Press, 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08754-05 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic Analysis of the Multidrug 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, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
12.0	12.0	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.)*

Resistance to multiple drugs is a major impediment to the successful chemotherapy of human cancers. To investigate the genetic and biochemical basis for this multidrug resistance (MDR) phenotype, we have developed a model system using the cultured KB cell, a human carcinoma cell line selected independently for resistance to high levels of either colchicine, adriamycin or vinblastine which is cross-resistant to colchicine, adriamycin, vincristine, vinblastine, puromycin, and actinomycin D. This resistance results from expression of the MDR1 gene which encodes a 170,000 dalton membrane glycoprotein (P-glycoprotein) which is a multidrug transport protein. ATP-dependent transport of vinblastine has been demonstrated using membrane vesicles from MDR cells. Expression vectors into which the MDR1 cDNA are cloned confer the complete MDR phenotype on drug-sensitive cells, as does a retrovirus carrying the MDR1 gene. Expression of MDR1 RNA and P-glycoprotein occurs in normal kidney, liver, colon, and adrenal and in tumors derived from these tissues which are intrinsically resistant to chemotherapy as well as several other tumors. Acquired drug-resistance in childhood leukemia, neuroblastoma, rhabdomyosarcoma and in pheochromocytoma may be associated with increased MDR1 RNA levels.

Other Professional Personnel:

C. Cardarelli	Research Biologist	LMB, NCI
M. C. Willingham	Chief, UCS	LMB, NCI
S. Kane	Guest Researcher	LMB, NCI
H. Galski	Visiting Fellow	LMB, NCI
L. Goldstein	Medical Staff Fellow	LMB, NCI
E. Bruggeman	Biotechnology Fellow	LMB, NCI
U. Germann	Guest Researcher	LMB, NCI
S. Currier	IRTA Fellow	LMB, NCI
P. Marino	Guest Researcher	LMB, NCI
F. Thiebaut	Visiting Fellow	LMB, NCI
S. Tanaka	Visiting Fellow	LMB, NIC
M. Horio	Visiting Fellow	LMB, NCI
K.-V. Chin	Visiting Fellow	LMB, NCI
R. Padmanabhan	Research Chemist	LMB, NCI
G. T. Merlino	Senior Staff Fellow	LMB, NCI
C. Jhappan	Visting Fellow	LMB, NCI

Major Findings:

1. Membrane vesicles prepared from MDR cell lines transport vinblastine in the presence of ATP and an ATP regenerating system. This transport is inhibited by the drugs vinblastine, daunomycin, actinomycin D and colchicine, and two drugs which reverse the MDR phenotype, verapamil and quinidine. The ATPase inhibitor vanadate is a non-competitive inhibitor of vinblastine transport.

2. To analyze the synthesis and processing of P-glycoprotein, and to detect its presence, we have prepared several rabbit polyclonal antibodies against synthetic peptides, and against polypeptide fragments synthesized in E. coli. One of the anti-peptide antibodies directed to a peptide near the amino terminus has been used to show that the protein has a long half-life (>48 hours) and is covalently modified by addition of phosphate and Asn-linked sugars.

3. Expression of a full-length MDR1 cDNA in drug-sensitive cells using eukaryotic expression vectors with the Ha-retroviral LTR promoter, themetallotheinein (MT) promoter, the SV40 (SV) promoter, or the β-actin (βAP) promoter, results in MDR cell lines. The MDR1 vectors can be used as dominantselectable markers to cotransfer and coamplify other genes, such as the gene for procathepsin L (MEP).

4. Transgenic mice carrying βAP-MDR, MT-MDR, and SV-MDR expression vectors have been constructed. One line of βAP-MDR mice has been studied in detail. Expression of human MDR1 mRNA has been found in several tissues of these transgenic mice including bone marrow and spleen.

5. A Ha-MDR retrovirus confers multidrug resistance on drug-sensitive cells with high efficiency. Localization of P-glycoprotein in MDCK (canine kidney) cells after Ha-MDR viral infection has been found on the apical surface of these polarized cells, indicating that the cDNA contains information for this specific localization.

6. Normal kidney, liver, colon, small intestine and adrenal express P-glycoprotein. In kidney, liver, colon and small intestine, P-glycoprotein is located on the apical (lumenal) surfaces of transporting epithelia, consistent with a transport function for this protein.

7. Deletions and premature termination mutations have been constructed in the MDR1 gene and their activity tested in the pHaMDR1 expression system. All tested deletions or premature terminations within the gene render it inactive, except for small deletions at the C-terminus.

8. The MDR1 gene is expressed in untreated human tumors including many tumors derived from the colon, kidney, adrenal gland, liver and pancreas in addition to chronic myelogenous leukemia in blast crisis. Occasionally elevated expression is found in other untreated tumors such as neuroblastoma, acute lymphocytic leukemia in adults, acute non-lymphocytic leukemia in adults, carcinoid tumors, indolent non-Hodgkins lymphoma and in cell lines of non-small cell carcinoma of the lung with neuroendocrine properties. Increased expression was detected in some tumors at relapse after chemotherapy including acute lymphoblastic leukemia of childhood, neuroblastoma, rhabdomyosarcoma, pheochromocytoma and nodular poorly differentiated lymphoma. Other untreated tumors usually express undetectable or low levels of MDR1 RNA; these include head and neck cancer, Wilm's tumor, esophogeal cancer, breast cancer, ovarian cancer, small cell carcinoma of the lung, and other non-small cell carcinomas of the lung. The evaluation of MDR1 expression in both intrinsic and acquired drug resistance may prove to be a valuable tool in the design of chemotherapeutic protocols.

9. MDR1 RNA can be detected semi-quantitatively by in situ hybridization analysis in MDR tissue culture cells. Loss of resistance occurs by segregation of amplified genes in a heterogeneous manner with small "islands" of MDR RNA positive cells remaining. In situ hybridization shows some promise as a technique for clinical detection of MDR1 RNA positive tumors in which expression of RNA might be heterogeneous.

Publications:

Pastan I, Gottesman MM. Multiple drug resistance in human cancer. N England J of Med 1987;316:1388-93.

Ueda K, Cardarelli C, Gottesman MM, Pastan I. Expression of a full-length cDNA for the human mdr1 (P-glycoprotein) gene confers multidrug resistance, Proc Natl Acad Sci USA 1987;84:3004-8.

Thorgeirsson SS, Huber BE, Sorrell S, Fojo A, Pastan I, Gottesman MM. Expression of the multidrug-resistant gene in heaptocarcinogenesis and regenerating rat liver, Science 1987;236:1120-7.

Cornwell MM, Tsuruo T, Gottesman MM, Pastan I. ATP-binding properties of P-glycoprotein from multidrug resistant KB cells, The FASEB J 1987;1:51-4.

Gottesman MM, Pastan I, Akiyama S-1, Fojo AT, Shen D-w, Ueda, K, Clark DP, Cardarelli CO, Richert ND, Willingham MC, Cornwell, MM, Chin JC, Chen C-j, Choi K, Soffir R, Roninson IB. Expression, amplification, and transfer of DNA sequences associated with multidrug-resistance. In: Tew K, Wooley P, eds. Mechanisms of Drug Resistance in Neoplastic Cells. Bristol-Meyers Symposium on Cancer Research. New York: Academic Press Inc, 1987;243-257.

Fojo A, Cornwell MM, Cardarelli CO, Clark DP, Richert N, Shen D-w, Udea, K, Willingham M, Gottesman MM, Pastan I. Molecular biology of drug resistance. Breast Cancer Res. Treat. (The Hague) 1987;9:5-16.

Willingham MC, Richert ND, Cornwell MM, Tsuruo T, Hamada H, Gottesman MM, Pastan Pastan I. Immunocytochemical localization of P170 at the plasma membrane of multidrug-resistant human cells, J Histochem Cytochem 1987;35:1451-6.

Fojo AT, Shen D-w, Pastan I, Gottesman MM. Intrinsic drug resistance in human kidney cancer is associated with expression of a human multidrug-resistance gene, J Clin Oncol 1987;5:1922-7.

Ueda K, Pastan I, Gottesman MM. Isolation and sequence of the promoter region of the human multidrug-resistance (P-glycoprotein) gene, J Biol Chem 1987;262:17432-6.

Gottesman MM, Pastan I. Resistance to multiple chemotherapeutic agents in human cancer cells, TIPS 1988;263:254-61.

Akiyama S-i, Cornwell MM, Kuwano M, Pastan I, Gottesman MM. Most drugs that reverse multidrug resistance also inhibit photoaffinity labeling of P-glycoprotein by a vinblastine analog, Mol Pharmacol 1988;33:144-7.

Roninson IB, Shen D-w, Chin JE, Choi K, Fojo A, Soffir R, Richert N, Gros P, Housman DE, Gottesman MM, Pastan I. Multidrug resistance in human cells: The role of the mdr1 gene, ICN-UCLA Symposium, 1988; in press.

Gottesman MM, Roninson IB, Pastan I. A molecular basis for multidrug-resistance in human cancer cells. In: Kessel D, ed. Resistance to Antineoplastic Drugs. New York: CRC Press, 1988; in press.

Cornwell MM, Pastan I, Gottesman MM. Binding of drugs and ATP by P-glycoprotein in human multidrug-resistant cells. In: Roninson IB, ed. Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells. New York: Plenum Publishing Corporation, 1988; in press.

Gottesman MM, Willingham MC, Thiebaut, F, Pastan I. Expression of the mdr1 gene in normal human tissues. In: Roninson IB, ed. Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells. New York: Plenum Publishing Corporation, 1988; in press.

Gottesman MM, Fojo A, Galski H, Pastan I. Expression of the multidrug-resistance gene in human cancer. In: Roninson IB, ed. Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells. New York: Plenum Publishing Corporation, 1988; in press.

Horio M, Gottesman MM, Pastan I. ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells, Proc Natl Acad Sci USA 1988;85: 3580-4.

Pastan I, Gottesman MM, Ueda K, Lovelace, E, Rutherford A, Willingham MC. A retrovirus carrying a MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells, Proc Natl Acad Sci USA 1988; in press.

Chabner BA, Gottesman MM. Multidrug resistance in cancer chemotherapy. William Guy Foundation Think Tank Meeting Report. JNCI 1988;80:391-4.

Shen D-w, Pastan I, Gottesman MM. Acquisition and loss of the human multidrug-resistance gene: in situ hybridization analysis, Cancer Res 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08756-01 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Transgenic Mouse as a Model System to Study Gene Function and Regulation

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

PI:	G. T. Merlino	Senior Staff Fellow	LMB, NCI
	I. Pastan	Chief, Laboratory of Molecular Biology	LMB, NCI
Other:	H. Galski	Visiting Fellow	LMB, NCI
	P. Marino	Guest Researcher	LMB, NCI
	C. Jhappan	Visiting Fellow	LMB, NCI
	M. M. Gottesman	Chief, Molecular Cell Genetics Section	LMB, NCI

COOPERATING UNITS *(if any)*

G. Jay, Chief, Cell Physiology Section, LMV	DCE, NCI
D. Lowy, Chief, Laboratory of Cellular Oncology	DCBD, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	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 production of transgenic mice constitutes a new and powerful approach to the study of gene function and regulation. We have used this new technology to begin to investigate two important aspects of cancer progression: the ability of malignant cells to escape destruction by chemotherapy, and the role of proto-oncogenes in the initiation of the transformed phenotype.

A gene (MDR1) encoding a multidrug transporter (P-170) capable of conferring multidrug resistance was microinjected into one-cell mouse embryos and germ line transgenic mice established. Expression of the MDR1 gene was detected in the normally drug-sensitive bone marrow. Studies are underway to determine if transgenic mice overexpressing the MDR1 gene in bone marrow are more resistant to chemotherapy.

The epidermal growth factor (EGF) receptor is the cellular homolog of the avian erythroblastosis virus erbB oncogene product. To elucidate the role that the EGF receptor plays in normal and transformed cells, we generated transgenic mice that carry germ line-integrated EGF receptor genes. These mice are being analyzed for EGF receptor gene expression, and scrutinized for the appearance of cancer.

GPO 914-918

Major Findings:

Transgenic mice have been generated by microinjecting various purified DNA fragments into one cell C57BL/6XSJL mouse embryos. The resulting embryos were reimplanted into pseudopregnant foster mothers. Tail DNA from two week old offspring was analyzed for foreign DNA integration. Mice containing the transgene were expanded and lines established.

A human gene has been isolated (MDR1) that encodes the 170 kDa-glycoprotein (P-170) capable of conferring multidrug resistance to animal cells. (See Annual Report Number Z01 CB 08754.) Several lines of transgenic mice were developed carrying the MDR1 cDNA driven by a variety of transcription promoters: (1) Three transgenic lines in which a chicken -actin gene promoter-MDR1 DNA fragment is inserted into the mouse genome. These mice are all being analyzed for expression of the MDR1 transgene. By RNA blot analysis, expression of RNA was detected in the bone marrow, which is normally especially sensitive to chemotherapeutic agents. Studies are presently underway to determine if transgenic mice overexpressing the MDR1 gene in bone marrow are more resistant to chemotherapy. (2) Seven lines of mice in which the MDR1 gene is expressed by way of the SV40 early promoter (in collaboration with G. Jay). (3) Two lines of mice where the MDR1 gene is driven by the metal-inducible mouse metallothionine gene promoter.

In collaboration with D. Lowy, we have found the NIH3T3 cells infected with a retrovirus carrying the EGF receptor coding region overexpress the receptor, undergo EGF-dependent transformation in tissue culture and form large tumors in nude mice. These results indicate that the overexpressed EGF receptor can contribute to the appearance of the transformed phenotype. To examine this question in vivo, we placed the EGF receptor gene under the control of the metallothionine promoter, and microinjected this DNA into one-cell mouse embryos. Five lines of this fourth type of transgenic mouse were developed carrying the EGF receptor DNA in their germ line. Experiments are in progress to determine the expression patterns of this transgene and the consequences of its overexpression in the developing and adult mouse.

In a related project, the promoter of the EGF receptor proto-oncogene has been isolated and is being characterized by protein-binding assays and mutagenesis analysis (see Annual Report Number Z01 CB 08000 for details). We plan to examine EGF receptor gene promoter function in vivo by generating transgenic mice containing DNA fragments consisting of the bacterial marker gene chloramphenicol acetyltransferase (CAT) driven by the normal EGF receptor gene promoter or by mutations of that promoter. I have specifically focused on a promoter site that is both sensitive to single stranded-specific nucleases and necessary for optimal cell-free transcription. This region contains binding sites for the positive transcription factor Sp1 and a factor (TCF) I am beginning to characterize. By examining and comparing the in vivo activity of normal and mutated promoters in a variety of mouse tissue types, we will elucidate the role of specific promoter sites in EGF receptor gene regulation.

Publications:

Velu TJ, Beguinot L, Vass WC, Willingham MC, Merlino GT, Pastan I, Lowy DR. EGF-dependent transformation by a human EGF receptor proto-oncogene, Science 1987;238:1408-10.

Johnson AC, Ishii S, Jinno Y, Pastan I, Merlino GT. EGF receptor gene promoter: Deletion analysis and identification of nuclear protein binding sites, J Biol Chem 1988;263:5693-9.

Merlino GT, Jinno Y, Johnson AC. Modulation of EGF receptor proto-oncogene transcription by a promoter site sensitive to S1 nuclease, Mol Cell Biol 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08757-01 LMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

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

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

PI:	D. J. FitzGerald	Microbiologist	LMB, NCI
Other:	I. Pastan	Chief, Laboratory of Molecular Biology	NCI
	M. C. Willingham	Chief, UCS	LMB, NCI
	S. Adhya	Chief, DGS	LMB, NCI
	T. Kondo	Visiting Fellow	LMB, NCI
	J. Pearson	Frederick Cancer Research Facility	NCI
	T. Waldmann	Chief, Metabolism Branch, DCBD	NCI
	M. Bookman	Clinical Oncology Prgm, Medicine Branch	DCT, NCI

COOPERATING UNITS *(if any)*
Biological Research Modifier Program
Metabolism Branch, NCI
Medicine Branch, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Ultrastructural Cytochemistry Section

INSTITUTE AND LOCATION
Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	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.)*

Initially, OVB3-PE was described as a disulfide conjugate with an ID_{50} for OVCAR-3 cells of 10-20 ng/ml, an LD_{50} for mice of 4 µg and antitumor activity that prolonged survival of tumor-bearing mice by 40 days. The same conjugate made by thioether linkage (OVB3-PE/s-c) is 10- to 20-fold more cytotoxic (ID_{50} 1 ng/ml) for OVCAR-3 cells and has an LD_{50} of 2 µg for mice. OVB3-PE/s-c, given to nude mice bearing the OVCAR-3 tumor increased the median survival by greater than 100 days. This result was seen when the immunotoxin was given 3-5 days after tumor cells had been implanted. Increased survival was also noted when mice were treated at much later times.

To obtain approval for Phase 1 clinical testing, OVB3-PE/s-c was evaluated for toxicity in monkeys. Some elevation in liver enzymes was noted.

OVB3-PE/s-c has been administered to four patients with recurrent ovarian cancer. Post-treatment sampling of peritoneal fluid showed that active immunotoxin was recovered from two of these patients. By measuring the recoverable immunotoxin activity over time, the kinetics of immunotoxin egress from the abdominal cavity was determined. The other two patients had neutralizing activity to PE. Because of this, a sensitive PE-neutralizing assay was developed to screen sera from all prospective patients.

The OVB3 antibody binds some colon and breast cancer cell lines. HT-29 and MCF-7 cell lines were killed by OVB3-PE/s-c. OVB3-PE/s-c had antitumor activity against HT-29 cells growing in the peritoneal cavity of nude mice. When used alone, OVB3-PE/s-c had a modest increase in survival but when used in combination with chemotherapy much greater survival was achieved.

Immunotoxins were also made using recombinant forms of PE such as PE40 and LysPE40. AntiTac-PE40 and HB21-PE40 were about 10-fold less active than the corresponding PE conjugates; however, antiTac-LysPE40 had equal potency to antiTac-PE.

GPO 914-918

Major Findings:

Previously, we showed that Pseudomonas exotoxin (PE) chemically coupled to the monoclonal antibody OVB3 by a disulfide bond could kill OVCAR-2 and OVCAR-3 cells with an ID_{50} of 10-20 ng/ml. This conjugate was also tested in a nude mouse model of ovarian cancer and found to have antitumor activity when injected intraperitoneally against OVCAR-3 tumors. The maximum tolerated dose was 2 µg per injection (three daily injections were given) and at this dose level median survival times of tumor-bearing mice were increased from 50 days for untreated mice to approximately 90 days for treated mice. Injections of 4 µg of OVB3-PE were lethal.

Recently, an active conjugate between OVB3 and PE was made by thioether linkage that had 10- to 20-fold greater cell-killing activity than the corresponding disulfide-linked immunotoxin. Bjorn et al. had described a method to make thioether conjugates that had similar cell-killing activities to PE-immunotoxins made by disulfide linkage. We chose an alternate method and a different cross-linker to make a novel thioether linkage between OVB3 and PE (OVB3-PE/s-c). The disulfide and thioether-linked immunotoxins were compared for cell-killing activity in tissue culture, lethality for mice and antitumor activity in the nude mouse model of ovarian cancer. The thioether immunotoxin had an ID_{50} of 1 ng/ml for OVCAR-3 cells compared to 10-20 ng/ml for the disulfide, a LD_{50} of 2 µg for 20g mice compared to 4 µg for the disulfide linkage and could increase the median survival to 150 days or longer compared to 90 days for the disulfide. Initially all experiments to assess antitumor activity were performed by injecting 50 x 10^6 tumor cells on day 1 and starting immunotoxin treatments 3-5 days later. The thioether linked immunotoxin was also tested against a much larger tumor burden. Mice were treated at 19 or 26 days post inoculation with tumor cells. At this time, the mice had gained 5-10g in body weight and thier abdomens were visibly distended. Five I.P. injections of OVB3-PE/s-c reduced the weights close to normal and also reduced abdominal swelling. While mice treated in this fashion were not cured, survival was prolonged by 25-30 days. Further analysis showed that the late treatments killed primarily unattached tumor cells but did not significantly reduce the size of solid implants.

Clinical lots of OVB3-PE/s-c were made in the laboratory and subsequently tested for general safety, pyrogenicity, and sterility. Toxicity tests in cynomolgus monkeys at doses of 0.05 and 0.01 mg/kg were also performed. The monkeys at both test doses remained grossly healthy but a transient elevation in liver enzymes was noted.

OVB3-PE/s-c received FDA approval for Phase I testing and four patients have been treated at a dose of .001 mg/kg. The immunotoxin was instilled in 2 liters of saline and samples were removed at various times to determine the half-life. Active immunotoxin was detected at times up to 12 hours post infusion in two of the four patients. The half-life was approximately 2 hours. Neutralizing antiPE antibodies were detected in the other two patients.

To determine the prevalence of antiPE antibodies in relevant patient populations, 165 serum samples were screened using a sensitive PE neutralization test. This revealed that 30% of sera provided by the BRMP from ovarian cancer patients, and previously screened for OC125 antigen, had neutralizing antibodies.

In contrast, samples provided by Mayo Clinic showed that no more than 3% of women with ovarian cancer, or non-malignant gynecologic disease or normal subjects had neutralizing antibody. We speculate that patients treated with experimental therapies and multiple procedures have a higher incidence of Pseudomonas infections and that the samples provided by the BRMP fell into this category.

The OVB3 antibody also binds to some colon and breast cancer cell lines. In tissue culture, HT-29 and MCF-7 cells were killed by OVB3-PE/s-c. OVB3-PE/s-c has antitumor activity against HT-29 cells growing in the peritoneal cavity of nude mice. When used alone, OVB3-PE/s-c had a modest increase in survival but when used in combination with chemotherapy much greater survival times were achieved.

AntiTac-PE has been administered to four patients with adult T-cell leukemia. The maximum tolerated dose of antiTac-PE for humans appears to be 4-5 mgs given over 3-4 days. This amount produced a small elevation in liver enzymes. For mice, 2-4 μg is lethal. To reduce in vivo toxicity for liver, antiTac was conjugated to recombinant forms of PE lacking the PE binding domain. Initially, antiTac-PE40, was made had an LD_{50} of ~100 μg for mice but was also 10-fold reduced in its ability to kill target HUT-102 cells. A second form of this conjugate was made following the addition of a lysine near the amino terminus of PE40. AntiTac-lysPE40 also had reduced toxicity for mice but was fully active in target cell-killing for HUT-102 cells when compared to antiTAC-PE.

Publications:

Kondo, T., FitzGerald, D., Chaudhary, V., Adhya, S., and Pastan, I.: Activity of immunotoxins constructed with modified pseudomonas exotoxin A lacking the cell recognition domain. J. Biol. Chem. (in press, 1988).

SUMMARY REPORT
LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE
October 1, 1987 to September 30, 1988

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 4-year residency program in anatomic pathology is provided for 9 residents and 3 fellows. The Laboratory is divided into 9 sections:

Surgical Pathology Section (Dr. Maria J. Merino, Acting Chief)
Pulmonary and Postmortem Section (Dr. William D. Travis, Acting Chief)
Cytopathology Section (Dr. Diane Solomon, Chief)
Ultrastructural Pathology Section (Dr. Timothy J. Triche, Chief)
Biochemical Pathology Section (Dr. David A. Zopf, Chief)
Tumor Invasion and Metastases Section (Dr. Lance A. Liotta, Chief)
Hematopathology Section (Dr. Elaine S. Jaffe, Chief)
Gene Regulation Section (Dr. David L. Levens, Acting Chief)
Office of the Chief (Dr. Lance A. Liotta, 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.

Surgical Pathology Section

Approximately 6,000 surgical specimens or biopsies (approximately 60,000 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 diagnostic immunohistology service is provided for clinical diagnosis and research. A tissue procurement nurse works closely with the staff and helps in the distribution of specimens to scientists. Clinicopathological studies in pediatric neoplasms, soft tissue sarcomas, acquired immune deficiency syndromes, mast cell disease, human breast cancer diagnosis, and lung pathology are in progress.

Dr. Merino is investigating the use of the thymidine labelling index (TLI) to study the growth fraction of human breast carcinomas over a range of histologic types, before and after therapy. The goal will be to correlate TLI with relapse rates and response to therapy.

Pulmonary and Postmortem Section

Dr. Travis is conducting a detailed review of interstitial fibrotic lung disease. Lung biopsies from 60 patients with idiopathic pulmonary fibrosis have already been reviewed and the data are currently being analyzed. Biopsies from 30 patients with pulmonary histiocytosis X are also being studied. The light microscopic findings observed in these lung biopsies are being correlated with clinical features, bronchoalveolar lavage data and ultrastructural findings.

Laboratory of Pathology, DCBD, NCI

The pulmonary and autopsy pathological material is being actively utilized by the staff and residents for research projects involving clinicopathological correlation and pathological characterization of diseases studied at the Clinical Center. Electron microscopic and immunocytochemical techniques are being applied to the study of these diseases. A comprehensive study of the pulmonary pathology of the Acquired Immune Deficiency Syndrome (AIDS) is currently being conducted based on surgical and autopsy lung pathology material from AIDS patients seen at the NIH.

The neuropathology service is integrated with the Surgical Pathology Section, Pulmonary and Postmortem Section, and the Ultrastructural Pathology Section. Within the Laboratory of Pathology, both diagnostic (patient care) service and teaching (of pathology residents) are provided. The service also functions in a collaborative manner to provide neuropathological support for a wide range of clinicopathologic investigations.

Cytopathology Section

This section provides diagnostic services in cytology (both exfoliative and fine needle aspiration). During the year 7,500 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. Diane Solomon has been applying immunoperoxidase techniques to cytological materials.

Special expertise is provided in aspiration cytology. The section provides a) a pathologist to perform the aspiration; b) a pathologist to instruct the clinician in aspiration technique; c) a cytotechnologist to assist the clinician; d) assistance to the radiologist during the aspiration of a deep tissue specimen.

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 200 cases were accessioned; over 150 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 factors which determine the biologic aggressiveness of alveolar rhabdomyosarcoma compared to embryonal rhabdomyosarcoma.

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. Clinical treatment protocols have been designed based on the diagnostic distinction between Ewing's sarcoma, neuroblastoma and peripheral neuroblastoma.

n-myc oncogene expression is an important factor in neuroblastoma prognosis and biologic behavior. n-myc has been sporadically reported in rare other childhood tumors. Further antibody detection methods have recently become available. Dr. Triche has used a rabbit polyclonal antibody to detect the n-myc protein in tissue sections of the common tumors of childhood. His group has further correlated these results with conventional Northern, Southern, and Western analysis of examples of each of these tumors. They find no evidence of n-myc amplification, expression, or protein accumulation in any of these tumors save neuroblastoma.

Another area of active investigation by Dr. Triche is the prognostic classification of childhood rhabdomyosarcoma. He had previously found that cells from 6 human rhabdomyosarcoma (RMS) cell lines when injected in the subcutis of nude mice retain their initial cytology (embryonal vs. alveolar). Moreover, his group found correlation between the histologic subtype (alveolar vs. embryonal) of RMS and the time/size of tumor growth in the subcutis of nude mice. In addition, all cell lines were found to express high levels of the c-myc oncogene, with or without c-myc amplification. n-myc was not expressed or amplified in any of the lines.

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.

Antibodies of the J606 murine V_H family, GAC (group A-streptococcal carbohydrate) (GAC-related V_H genes), have been seen to derive in normal mice from at least $3V^H$ gene segments (of a locus whose complexity is 10-12). Two of

these are GAC-related, one inulin related. In CBA/N mice, which do not respond to either GAC or inulin by conventional immunization methods, antibodies of the J606 V_H family origin were found to constitute about 5% of their B cell V_H pre-immune repertoire. The extent of expression of the members of the J606 V_H family has been examined by nucleotide sequencing of hybridoma derived V_H J606 genes from their purified mRNA.

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 metalloproteinases which cleave type IV collagen of the extracellular matrix (type IV collagenase), b) a new type of matrix receptor (laminin 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 consistently 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. Monoclonal antibodies to the laminin receptor have been used to target adriamycin containing liposomes specifically to laminin receptor positive tumor cells.

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, their regulation and how they interact with the tumor cell, Dr. Sobel and other members of the section have isolated and characterized 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 with high affinity. They isolated a human laminin receptor cDNA and showed that laminin receptor mRNA in the tumor cell is a rate-limiting control step in the biosynthesis of the receptor, and hence in the regulation of cellular attachment to basement membranes via laminin. During the past year, they have extended the sequence analysis of the laminin receptor gene. In particular, they have obtained more amino terminal sequences. They discovered that there is more than one laminin receptor gene which may encode more than one protein expressed in the cell. They have also cloned a murine laminin receptor cDNA and have found that murine cells, like their human counterparts, have multiple laminin receptor genes. Studies of the regulation of the laminin receptor gene indicate that differentiated cells express less laminin receptor mRNA than do related, undifferentiated precursor cells. This is consistent with the general observation that aggressive undifferentiated tumors which metastasize express more laminin receptor.

Laboratory of Pathology, DCBD, NCI

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. Type IV collagenase was found to be markedly augmented following ras oncogene transfection.

Type IV collagenase was purified and subjected to amino acid sequencing. The primary sequence information was used to generate affinity purified anti-peptide antibodies which selectively recognize the latent versus the active form of the enzyme. These antibodies have been employed in the development of an ELISA assay for human serum samples. The antibodies were also used to screen a lambda gtll expression library and isolate a complete cDNA clone encoding human type IV collagenase. Significant progress has been made in the understanding of the mechanism of activation and the substrate cleavage site.

Activated ras oncogene transfection into suitable recipient cells has been shown by members of the section to induce the metastatic phenotype. With R. Pozzatti and G. Khoury, members of the section extended this work to ras^H transformed rat embryo cells. These cells were also highly metastatic when transformed either by ras^H or by ras^H linked to an enhancer. However, when the cooperating oncogene Ela was used in conjunction with ras^H, metastasis was only rarely seen. This model system was then used to study the correlation of basement membrane collagenolysis with metastatic propensity. The c-Ha-ras oncogene alone, or combined with v-myc, transfected into early passage rat embryo fibroblasts, induce these cells to secrete high levels of type IV collagenolytic metalloproteinase and to concomitantly exhibit a high incidence of spontaneous metastases in nude mice. Cotransfection of c-Ha-ras plus the adenovirus type 2 Ela gene yields cells which are highly tumorigenic but nonmetastatic and fail to produce type IV collagenase. Regulation was found to occur at the level of type IV collagenase mRNA.

The nonmetastatic cells which failed to produce type IV collagenase retain the ability to secrete high levels of plasminogen activator. Transfection with the proto-oncogenic forms of Ha-ras or mos, or spontaneous transformation of NIH 3T3 cells or chemical transformation of BALB 3T3 cells yields cells which fail to produce collagenase, are tumorigenic, but totally nonmetastatic. These data support a genetic linkage of type IV collagenase expression with the metastatic phenotype in this rodent system.

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 discovered that a number of metastatic cell lines produce and respond to autocrine motility factors. AMF was purified from the conditioned media of a human melanoma cell line and 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. AMF also acts through "G" proteins since pertussis toxin, an inhibitor of G protein action, markedly reduces AMF-stimulated motility. 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 much less 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. A cDNA clone for human AMF is being characterized. Furthermore, the measurement of AMF in urine samples appears to be an accurate marker of transitional cell carcinoma of the bladder.

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 and in vitro translation studies of paired nonmetastatic-metastatic cells revealed differences in the synthesis of specific proteins. A cDNA library was constructed from a highly metastatic murine melanoma cell line and was screened for clones that hybridized differentially to labeled RNAs from high and low metastatic melanoma cell lines. One clone, pNM23, recognizes two mRNAs present to a significantly greater degree in two low metastatic melanoma cell lines than in five related, highly metastatic cell lines. This cDNA probe also hybridizes to RNA extracted from subcutaneous tumors produced by the low metastatic melanomas to a greater extent than to tumors from five related, highly metastatic melanoma lines. The DNA sequence of the cDNA insert was obtained, and computer analysis of the predicted amino acid sequence of the only open reading frame reveals that the predicted gene product of the NM23 gene is a novel protein. The pNM23 cDNA probe thus identifies mRNAs differentially expressed by cells of varying metastatic potential both in vitro and in vivo. Further, metastases may not result simply from the acquisition of invasive and other traits, but may also involve the loss of certain cell functions. The level of expression of pNM23 showed a highly significant inverse correlation with human breast carcinoma aggressiveness measured by numbers of axillary lymph nodes positive for metastases. pNM23 is therefore a putative suppressor gene.

Hematopathology Section

The Hematopathology Section conducts a major program in diagnostic and experimental hematopathology. Dr. Jaffe supervises an internationally recognized consultation service for diagnostic hematopathology. 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.

Dr. Jaffe has continued to study the pathologic features of HTLV-I associated lymphomas. 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. Prospective studies of all lymphomas in similar geographic regions with differing incidences of adult T cell leukemia/lymphomas are studies to discern factors which make an impact on the incidence of HTLV-I and HTLV-I associated diseases.

Lymphocytes activated with IL2 both in vivo and in vitro are being used for the experimental treatment of human malignancies. The mechanism of action of this therapy is not clearly established, and it is not known whether those lymphokine-activated killer cells (LAK) infused actually migrate and infiltrate tumors, or whether other effector cells mediate the observed tumor regression. In order to better understand the pathophysiology of LAK-IL2 treatment, tumor biopsy specimens are obtained before, during, and after conclusion of LAK-IL2 treatment. Specimens are studied with an immunohistochemical technique for a battery of tumor markers as well as markers capable of identifying T cells, B cells, histiocytes, NK cells, and other effector cells. These data are then correlated with conventional, clinical and pathologic data to determine whether the immunopathology observed can predict clinical response.

Dr. Cossman has been studying 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.

Dr. Cossman's group has determined the incidence with which the t(14;18) chromosomal translocation involves each of the known DNA breakpoint clusters of the bcl-2 gene in a series of 85 cases of follicular lymphomas. Four breakpoint clusters were identified and the location of the breakpoint may influence the course of disease. Despite t(14;18) translocation, a substantial group (15%) was identified which did not rearrange bcl-2 loci in any of these breakpoint regions, indicating that additional breakpoints remain. He is attempting to clone the DNA sequences at new breakpoint regions and link them to the bcl-2 transcriptional unit.

The recently discovered gene encoding the delta chain of the second human T cell receptor lies within an actively rearranging region of the T cell receptor alpha locus. Using DNA probes of this region, his group has identified T-delta gene rearrangement as a frequent, early event in precursor T-cell neoplasms (ALL). Prior to our analysis, only one delta variable (V) gene was known. In

the course of their study, they have discovered two new productive V-delta genes and together the three V genes may comprise the entire delta V repertoire.

In an attempt to identify DNA sequences at the site of chromosomal translocation in Hodgkin's disease, he has cloned and sequenced the rearranged genomic DNA region of both the immunoglobulin heavy chain gene and T cell receptor beta chain gene in the Hodgkin's disease cell line, L428.

Dr. Cossman has demonstrated that frequent relapse of follicular lymphoma, the major obstacle to cure, is a consequence of clonal expansion of daughter cells derived from a common stem cell. Thus, despite the "clinical" remission achieved by therapy in most patients, residual lymphoma cells must persist. To detect occult lymphoma, he has specifically amplified the joined bcl-2/JH DNA sequences created by the t(14;18) translocation seen in nearly all follicular lymphomas. Using multiple rounds of primer-directed DNA polymerization (polymerase chain reaction, PCR), he can detect 1 copy of bcl-2/JH, which is four orders of magnitude more sensitive than flow cytometry or Southern blot restriction analysis. Genomic DNA sequence analysis of four lymphomas confirmed that the size of the amplified fragment serves as a unique tumor marker. Direct application to clinical samples has demonstrated lymphoma cells which were otherwise undetectable.

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 and oncogenes.

He has found that 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.

Since probes to the transferrin receptor gene have recently become available, Dr. Neckers is utilizing molecular biology techniques to study the regulation of transferrin receptor expression at the molecular level. One part of this project involves the study of mouse plasmacytoma mRNA. He has found that there are two transferrin receptor mRNAs in these cells. One message lacks the untranslated part of the full-length mRNA, while retaining the coding sequences for the protein. Using this model system, he plans to study the ability of iron to regulate TfR mRNA levels at the level of the message.

Dr. Neckers' group has produced a TfR construct which contains only the coding region of the cDNA under LTR control. They have successfully transfected 3T3 cells with this construct and have demonstrated the existence of human TfR on their surface by immunoprecipitation and FACS analysis. They will use these cells to study the regulation of the virally controlled TfR sequence.

Dr. Neckers has found that HTLV-I infected T cells express 10-20 fold more TfR on their surface, but that this is due to a redistribution of the receptor from cytoplasm to surface and not to an increased receptor synthesis. Further, the TfR in these cells is poorly internalized and poorly phosphorylated by phorbol

ester. The receptor cannot deliver iron to these cells, which nevertheless require iron for proliferation. Antibodies which prevent iron binding to the TfR still inhibit the growth of these cells.

Using an antisense c-myc oligomer, Dr. Neckers has shown that induction of IL-2 receptors, transferrin receptors, DNA polymerase α and blastogenesis in mitogen-treated T cells does not require c-myc protein. He went on to demonstrate that c-myc protein induction is not necessary for $G_0 \rightarrow G_1$, traversal, as was previously thought, but that c-myc protein is required for DNA synthesis. Dr. Neckers' group is the first to demonstrate the feasibility of using antisense oligomers to study the role(s) of specific c-myc nuclear proteins in cell activation and proliferation. Using an in vitro DNA polymerase α assay system, he has determined that both c-myc protein and a nuclear protein termed K_i-67 are required for DNA polymerase α activity.

Gene Regulation Section

Dr. David Levens has developed a novel method for identifying sequence-specific DNA binding proteins.

The gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with cellular proteins. These same proteins also bind to a negative cis-element approximately 330 bp upstream of c-myc promoter P1. The gibbon T-cell lymphoma cell line MLA 144 strongly transactivates the GALV-Seato enhancer. Fractionation of MLA 144 extracts by conventional and affinity chromatography allows the separation of the GALV enhancer binding protein complex into 2 components. One of these components interacts strongly with DNA but does not in itself possess full specificity. The second component does not in itself bind DNA, but upon forming a complex with the first component confers greatly enhancer power to discriminate between different sequences.

Application of an exonuclease assay developed in his laboratory to identify and map the sites of tight protein-DNA interactions on large pieces of DNA, to the c-myc has revealed multiple cis- and trans-elements both upstream and downstream of the c-myc promoter P1. The group has focused on 3 regions, each of which interacts with one or more sequence-specific binding proteins. A negative cis-element present in intron one of the c-myc gene is frequently mutated in Burkitt's lymphoma.

Office of the Chief

Drs. Davis, Larner and Mackem are young investigators who are designated under the Office of the Chief.

Dr. Mackem is investigating genes which regulate morphogenesis. The elucidation and characterization of genes necessary for establishing pattern formation

during morphogenesis and the study of their regulation are problems which are central to many aspects of vertebrate biology. It may be expected that processes including responses to trophic stimuli, cell-cell interactions, migration, differential cell mutliplication, programmed cell death, etc. are all involved. Many of these same processes are recapitulated in a pathologic manner during oncogenesis.

Dr. Davis is developing a system to study regulatory events in leukocyte adhesion and migration. HL-60 promyelocytic leukemia cells are known to be deficient in cell-substrate adhesive ability unless treated with differentiating agents which can induce either a granulocyte-like or monocyte-like phenotype. Dr. Davis has treated HL-60 cells with various known differentiating agents such as phorbol esters (TPA), dimethylsulfoxide (DMSO), 1,25 dihydroxyvitamin D3 (vitamin D3), dibutyryl cyclic AMP and retinoic acid and have examined the development of cell-substrate adhesive and migratory ability as well as the presence of proteolytic activities. The HL-60 cells can be induced to become adhesive and migratory with individual or combinations of differentiating agents mimicking the behavior of differentiated leukocytes. In addition, the cells release a 94 kDa gelatin-degrading metalloprotease which is induced during the development of cell-substrate adhesive ability with phorbol esters.

Dr. Larner is interested in the mechanism by which interferons regulate gene expression. He has isolated two cDNAs which correspond to two mRNAs whose expression is induced in an all or none fashion by type I interferons in a variety of human cultured cells. The induction of these mRNAs is mediated by an increase in the rate of transcription of these genes. The regulation of the transcription of the genes is complex, and involves both positive factors whose expression does not require new protein synthesis, and negative elements which inhibit the expression of these genes in certain types of cells after prolonged exposure to interferon. The action of these negative regulatory factors requires the synthesis of new proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00853-35 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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: M.J. Merino Acting Chief, Surgical Pathology Section LP NCI

OTHER: (see next page)

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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 Section provides service in anatomic pathology for the Clinical Center patients and collaborates with the research staff of all institutes in those investigations which involve the use and study of human pathological material. The 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 immunohistochemical 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 final stage. This will include space allocated for performance of special stains and immunocytochemistry.

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

Other Professional Personnel:

P. Cohen	Sr. Staff Fellow	LP NCI
W. Travis	Expert	LP NCI
M. Raffeld	Sr. Staff Fellow	LP NCI
S. Kennedy	Visiting Associate	LP NCI
+S. Pittaluga	Visiting Associate	LP NCI
+G. Jaffe	Staff Fellow	LP NCI
+L. Al-Gwaiz	Staff Fellow	LP NCI
+S. Vande Pol	Staff Fellow	LP NCI
+M Sadofsky	Staff Fellow	LP NCI
+A.C. Larner	Staff Fellow	LP NCI
+G. Davis	Staff Fellow	LP NCI
+K. Schmidt	Staff Fellow	LP NCI
+A. Ginsberg	Staff Fellow	LP NCI
*J. Stern	Consultant in Dermatopathology	

Objectives:

The objectives of the Surgical Pathology Section are:

(a) to provide diagnostic services in pathologic anatomy to the clinical research projects conducted at NIH;

(b) to carry out independent research;

(c) to provide a residency program in anatomic pathology; and

(d) to collaborate with investigators in research involving the use and study of human materials.

The proposed course of research includes (a) continuing to provide the services described; (b) increasing the interaction with the clinical branches in the design and evaluation of protocols; (c) improving the opportunities for the resident staff to participate in teaching, conferences, and seminars, and providing elective periods to be spent accomplishing research projects with the senior staff; and (d) implementing data retrieval programs.

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.
*This Associate Pathologist spends part time in the activities of the Surgical Pathology Section.

Publications:

Axiotis CA, Merino MJ, Lippes HA, Lanerolle NC, Stewart AF, Kinder B. Corticotroph cell pituitary adenoma within an ovarian teratoma. A new cause of ectopic ACTH syndrome. Am J Surg Pathol 1987;11:218-4.

Cromack DT, Sporn MB, Roberts AB, Merino MJ, Dart LL, Norton JA. Transforming growth factor - beta levels in rat wound chambers. J Surg Res 1987;42:622-8.

Sumpio BE, Jennings TA, Sullivan PD, Merino MJ. Adenoid cystic carcinoma of the breast. A review of 14 cases from the Connecticut Tumor Registry. Ann Surg 1987;205:295-1.

Ohuchi N, Page DL, Merino MJ, Viglione MJ, Kufe DW, Schlom J. Expression of tumor-associated antigen (DF3) in atypical hyperplasias and in situ carcinomas of the breast. J Natl Cancer Inst 1987;79:109-7.

Merino MJ. Small cell carcinoma of the uterus producing paraneoplastic syndrome. Am Assoc Clin Pathol (in press)

Kennedy S, Merino MJ. Collecting duct adenocarcinoma of kidney. Am Soc Clin Pathol. Check Sample Program, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09145-04 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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 Section, Pulmonary and Postmortem Section, and Ultrastructural Pathology Section, LP, NCI

LAB/BRANCH

Office of the Clinical Director, NINCDS

SECTION

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, MD 20892

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.)*

As described previously, 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, Postmortem, and the Ultrastructural Pathology Sections. Within the Laboratory of Pathology, both diagnostic (patient care) service and teaching (of pathology residents) are provided. The service also functions in a collaborative manner to provide neuropathological support for a wide range of clinicopathologic investigations.

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

Major Findings:

The neuropathology service continues to function: (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: As in previous years, the brain was examined in approximately 75% of all autopsies, 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: Similar to that described previously. Approximately 200 neurosurgical specimens are examined yearly, including both submitted and in-house cases. Approximately 50 intra-operative frozen section consultations are provided yearly. Current in-house case material includes pituitary adenomas, stereotactic biopsies (tumors, AIDS-related lesions), electrocorticographically guided resections for temporal lobe seizures, and PML (progressive multifocal leukoencephalopathy).

Conferences: The case material described above is also utilized for resident teaching conferences and neurology conferences, including presentations at NINCDS Grand Rounds (both formal CPC's and subject reviews).

Specific Studies:

1. Dementia: autopsy confirmation and clinical correlation of patients clinically diagnosed as having Alzheimer's disease (NIA, NIMH, NINCDS).

2. Pituitary adenomas: retrospective study of pituitary adenomas in Cushing's disease.

3. Progressive multifocal leukoencephalopathy (PML): rapid diagnosis/correlation of immunoperoxidase, in-situ hybridization, and EM findings; pathogenesis, including development of an animal model.

4. Malignant gliomas: pathologic study of effects of implanted radiation seeds for malignant gliomas; review of experimental and human use of IL2-LAK for malignant gliomas.

Publications:

Wanke C, Tuazon CU, Kovacs A, Dina T, Davis DO, Barton NB, Katz D, Lunde M, Levy C, Conley FA, Lane HC, Fauci AS, Masur H. Toxoplasma encephalitis in patients with AIDS: diagnosis and response to therapy. Am J Trop Med Hyg 1987;36:509-6.

DeChiro G, Hatazawa J, Katz DA, Rizzoli HV, DeMichele D. Glucose utilization by intracranial meningiomas as an index of tumor aggressivity and probability of recurrence: a positron emission tomography study. Radiology 1987;164:521-6.

Jackson D, Kinsella T, Rowland J, Wright D, Katz D, Main D, Collins J, Glatstein E. Hologenated pyrimidines as photosensitizers in the treatment of glioblastoma multiforme. Am J Clin Oncol 1987;10:437-4.

DiChiro G, Oldfield E, Wright DC, DeMichele D, Katz DA, Patronas N, Doppman JL, Larson SM, Ito M, Kufta CV. Cerebral necrosis after radiotherapy and/or intraarterial chemotherapy for brain tumors: PET and neuropathologic studies. Am J Neurol Res 1987;8:1083-1.

Houff SA, Major EO, Katz DA, Kufta CV, Sever JL, Pittaluga S, Roberts JR, Gitt J, Saini N, Lux W. Involvement of JC-virus-infected mononuclear cells from the bone marrow and spleen in the pathogenesis of progressive multifocal leukoencephalopathy. N Eng J Med 1988;318:301-5.

Kinsella TJ, Collins J, Rowland J, Klecker R, Wright D, Katz D, Steinberg S, Glatstein E. Pharmacology and phase I/II study of continuous intravenous infusions of iododeoxyuridine (IdUrd) and hyperfractionated radiotherapy in patients with glioblastoma multiforme. J Clin Oncol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09154-02 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Prognostic Significance of Thymidine Labelling Index in Breast Cancer

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

PI:	M.J. Merino	Acting Chief, Surgical Pathology Section	LP NCI
OTHER:	S. Kennedy	Visiting Associate	LP NCI
	S. Swaine	Senior Staff Fellow	MB NCI
	M. Lippman	Head, Medical Breast Cancer Section	MB NCI

COOPERATING UNITS *(if any)*

Medicine Branch

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1	.25

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 main purpose of the study will be to determine whether the thymidine labelling index could provide any significant information regarding prognosis in breast carcinoma, and to what extent the TLI could identify those patients that could have an early relapse. Until now, histologic type of the tumor and steroid receptor status have played an important role in the planning of adjuvant therapy, but we believe that the proliferation characteristics of tumor cells may also be a critical determinant since nuclear DNA contents in populations of breast cancer cells have been proven to have prognostic significance.

The thymidine labelling technique relies on the uptake of titrated thymidine by replicating cells which is then detectable by autoradiography. We will investigate the thymidine labelling index range for different histologic types of breast cancer, tumors of patients that have received radiation and/or chemotherapy; the changes in TLI of residual normal breast tissue and the effect of hormonal manipulation in TLI.

The rapid processing of autoradiographs allows cell kinetic data for clinical evaluation in three days, which may be a useful adjunctive diagnostic tool on selected cases.

PHS 6040 (Rev. 1/84)

GPO 914-916

Major Findings:

1. We will try to determine whether the thymidine labelling index could provide information regarding prognosis in breast carcinoma.

2. We will study breast tissues of patients that have previously received radiation or chemotherapy and compare the thymidine labelling index with untreated tumors and with previous biopsies.

Publications:

Duray PH, Merino MJ, Mark EJ. Metaplastic and osteoclastic breast carcinoma. An enzyme histochemical analysis of the multinucleated giant cells. Am J Clin Pathol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09165-01 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Pulmonary and Postmortem Pathology

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

PI: W.D. Travis Acting Chief, Pulmonary and Postmortem Pathology Section LP NCI

OTHER: Carl Baker, Paul Cohen, Peter Howley, David Levens, Lance Liotta, Susan Mackem, Timothy O'Leary, Layla Al-Gwaiz, George Davis, Ann Ginsberg, Gitie Jaffe, Andrew Larner, Stefania Pittaluga, Karen Schmidt, Scott Vande Pol, Lois Ritchie, Alex Dock, James Rainey, William C. Roberts, and David A. Katz.

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Pulmonary and Postmortem Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
22	19	3

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 Section of Pulmonary and Postmortem Pathology, together with the Cytopathology Section, Hematopathology Section, Surgical Pathology Section, and Ultrastructural Pathology 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. The Autopsy Suite was recently renovated and new equipment specially designed for safety was developed in conjunction with the Occupational Safety and Health Branch, Division of Safety, Office of the Director. A set of special autopsy safety policies, many of which were developed in our department, is utilized to protect our staff and residents from exposure to tissues contaminated with high risk infectious agents and radiation.

The pulmonary and autopsy pathological material is being actively utilized by the staff and residents for research projects involving clinicopathological correlation and pathological characterization of diseases studied at the Clinical Center. Electron microscopic and immunocytochemical techniques are being applied to study of these diseases. A comprehensive study of the pulmonary pathology of the Acquired Immune Deficiency Syndrome (AIDS) is currently being conducted based on surgical and autopsy lung pathology material from AIDS patients seen at the NIH.

Objectives:

The objectives of the Pulmonary and Postmortem Section are: (a) to provide diagnostic services in pulmonary and autopsy pathology for the clinical research projects conducted at NIH. This includes generating pathology reports for current cases and presenting the pathologic findings of autopsies and lung biopsies at clinical conferences with clinicians and radiologists; (b) to carry out independent research; (c) to provide teaching to the NIH residency program in anatomic pathology; and (d) to collaborate with investigators in research involving the use and study of human materials.

The proposed research program includes (a) continuing to provide the services described; (b) increasing interaction with clinical branches in the design and evaluation of protocols; (c) providing opportunities for residents to participate in teaching, and in research projects; (d) developing data retrieval systems for the autopsy and pulmonary pathology material.

Publications:

Travis WD, Banks PM, Reiman HR. Primary malignant lymphoma (Stage IA_E) of the soft tissues and extremities, a clinicopathologic study of eight cases. Am J Surg Pathol 1987;11:359-6.

Travis WD, Li C-Y, Banks PM, Nichols WL. Megakaryoblastic transformation of chronic granulocytic leukemia. Cancer 1987;60:193-0.

Travis WD, Wold LE. Immunoperoxidase staining of fine needle aspiration biopsies. Acta Cytol 1987;31:517-0.

Bauer S, Alpert L, Carski T, Clausen J, Daniel A, DiSalvo A, Conlon J, Fallon K, Favero M, Hadley W, Holland P, Ishimaru K, Luebbert P, Macher A, Neimaster R, O'Brien T, Ohlemeier J, Perna V, Robin H, Saah A, Safai B, Saw D, Schochetman G, Thomann W, Travis W, Young C. National Committee for Clinical Laboratory Standards. Protection of Laboratory Workers from Infectious Disease transmitted by Blood and Tissue, Proposed Guideline. NCCLS Document M29-P, Vol 7, No 9, Villanova, PA, 1987.

Travis WD, Li C-Y. Pathology of the lymph node and spleen in systemic mast cell disease. Mod Pathol 1988;1:4-14.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09166-01 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Pathology of Interstitial Pulmonary Fibrosis

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

PI:	W.D. Travis	Acting Chief, Pulmonary and Postmortem Pathology Section	LP NCI
OTHER:	V. Ferrans	Chief, Ultrastructure Section	IR PA NHLBI
	H.A. Jaffe	Senior Investigator	IR PB NHLBI
	R.G. Crystal	Chief, Pulmonary Branch	IR PB NHLBI

COOPERATING UNITS *(if any)*

Pulmonary Branch, NHLBI

LAB/BRANCH

Laboratory of Pathology

SECTION

Pulmonary and Postmortem Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	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.)*

A detailed pathologic review of the NIH (Pulmonary Branch, NHLBI) experience with pulmonary interstitial fibrosis is being performed to investigate potential new approaches to the diagnosis and pathologic subclassification of interstitial fibrosis. Recent reports have described newly recognized forms of interstitial fibrotic lung disease previously classified as idiopathic pulmonary fibrosis suggesting a need for rethinking of traditional concepts of the pathology of pulmonary interstitial fibrosis.

The broad experience of the Pulmonary Branch, NHLBI, provides a rich resource of clinical and pathologic material which may provide the basis for recognition of new prognostically significant forms of interstitial lung fibrosis.

Lung biopsies from 60 patients with idiopathic pulmonary fibrosis have already been reviewed and the data are currently being analyzed. Biopsies from 30 patients with pulmonary histiocytosis X are also being studied. The light microscopic findings observed in these lung biopsies are being correlated with clinical features, bronchoalveolar lavage data and ultrastructural findings.

GPO 914-918

Publications:

Travis WD, Carpenter HA, Lie JT. Diffuse pulmonary hemorrhage: an uncommon manifestation of Wegener's granulomatosis. Am J Surg Pathol 1987;11:702-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00852-35 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

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:	D. Solomon	Chief, Cytopathology Section	LP NCI
	L. Elwood	Senior Staff Fellow	LP NCI
	T.A. Wood	Biologist	LP NCI
	L. Galito	Biologist	LP NCI
	A.M. Wilder	Biologist	LP NCI
	E. Sanders	Bio. Lab. Tech.	LP NCI
	K. Condron	Microbiologist	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7	3	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 and fine needle aspiration cytology. The section also routinely applies immunocytochemistry techniques to confirm and/or enhance cytological diagnostic accuracy. In addition, the section collaborates in various clinical research projects utilizing routine microscopy as well as special staining techniques, immunocytochemistry, and flow cytometry.

The fine needle aspiration service is designed to afford maximal flexibility for clinicians and patients. Clinicians may request:

1) a pathologist perform the aspiration
2) a pathologist instruct the clinician in aspiration technique
3) a cytotechnologist assist the clinician in handling the specimen
4) aspirations of deep lesions performed by the radiologist with the assistance of a cytotechnologist to evaluate adequacy of the specimen

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

Approximately 3400 cytology specimens were evaluated over the past year in the Cytopathology Section. Diagnoses are generally available within 24 hours of receipt. Preliminary diagnoses on STAT cases are communicated within 1-2 hours. Cytology is no longer simply a screening modality: Cytologic evaluation often provides definitive diagnoses which dictate patient care and treatment.

Fine needle aspiration specimens have increased in number by approximately 85% over previous years. This modality has been embraced by clinicians as a minimally invasive technique which provides diagnoses rapidly and cost effectively, with minimal discomfort to the patient often obviating more invasive biopsy procedures.

Cases submitted by outside pathologists for consultation by the Cytopathology Section have increased in number by approximately 500% over previous years.

Cytological techniques are utilized in collaborative work with other sections and branches of NIH. For example, single cell tumor suspensions, tumor cell lines, and stimulated lymphocyte cultures are evaluated microscopically and by immunocytochemical techniques.

Publications:

Solomon D, Schwartz A. Renal pathology in Von Hippel-Lindau disease. Hum Pathol (in press)

Solomon D, Merino M, Linehan M, Jaffe E. Angiocentric malignant lymphoma involving lung and kidney: Mimicking renal cell carcinoma in fine needle aspiration cytology. Surg Pathol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00897-05 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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:	D. Solomon	Chief, Cytopathology Section	LP NCI
OTHER:	E.S. Jaffe	Chief, Hematopathology Section	LP NCI
	L. Elwood	Senior Staff Fellow	LP NCI
	K. Condron	Microbiologist	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.5	.25	.25

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.

We are extending the utilization of lymphoid markers to fine needle aspiration specimens of lymph nodes. Fine needle aspiration may obviate the need for repeat biopsies in patients with recurrent lymphoma.

GPO 914-916

Major Findings:

We have investigated 240 specimens, including 123 pleural effusions, 26 ascites, 38 cerebrospinal fluids, and 53 fine needle aspiration specimens. We have found 110 cases to be positive for lymphoma and 88 to be reactive in nature. Of the 110 positive cases, 87 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 21 cases on the basis of aberrant marker phenotype or TdT positivity. Acute nonlymphocytic leukemia was diagnosed in one case.

The application of immunocytochemistry to cytology specimens is an extremely valuable adjunct in the diagnosis of hematopoietic malignancies. Definitive cytological diagnosis of relapse/recurrence of disease guides clinical treatment of these patients, often obviating more invasive diagnostic procedures.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09153-02 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Cytophenotypic Analysis of Tumor Suspensions and TIL Cultures in Immunotherapy

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

PI:	D. Solomon	Chief, Cytopathology Section	LP NCI
OTHER:	S. Topalian	Fellow	SB NCI
	S. Rosenberg	Chief	SB NCI
	K. Condron	Microbiologist	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.75	.25	.50

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.)*

Clinical trials employing the adoptive transfer of expanded tumor infiltrating lymphocytes to patients with metastatic disease are currently underway under the direction of the Surgery Branch, NCI. Our collaborative effort in this project involves immunocytochemical analysis of tumor cell suspensions to identify (1) the percentage and phenotypic expression of subsets of tumor infiltrating lymphocytes present in the tumor and (2) tumor markers, if any, which are expressed by the tumor cells. Once the tumor infiltraing lymphocyte cultures have been expanded and are to be harvested for patient therapy, we analyze the material using routine cytologic preparations and immunocytochemistry to ensure the cultures are free of tumor cells.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

One hundred twelve tumor suspensions have been evaluated for tumor associated antigens and for phenotypic analysis of tumor infiltrating lymphocytes including: melanomas, renal cell carcinomas and sarcomas.

Seventy-five tumor infiltrating lymphocyte cultures have been examined. In four cases, rare tumor cells were identified. Seventy-one cultures were free of detectable tumor cells. The majority of reactive lymphoid cells from TIL cultures are Leu-4 positive.

Publications:

Topalian S, Muul L, Solomon D, Rosenberg S. Expansion of human tumor infiltrating lymphocytes for use in immunotherapy trials. J Immunol Meth 1987;102:127-41.

Topalian SL, Solomon D, Frederick FP, Chang AE, Freerksen DL, Linehan WM, Lotze MT, Robertson CN, Seipp CA, Simon P, Simpson CG, Rosenberg SA. Immunotherapy of patients with advanced cancer using tumor infiltrating lymphocytes and recombinant interleukin-2: A pilot study. J Clin Oncol 1988;6:839-53.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00545-10 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on 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 20892

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.

This project is now complete.

PHS 6040 (Rev. 1/84)

GPO 914-918

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.

Publications:

Scarpa S, Modesti A, Triche TJ. Extracellular matrix synthesis by undifferentiated childhood tumor cell lines. Am J Pathol 1987;129:74-85.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09137-04 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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)*

Dr. Samuel Navarro, U.S.-Spain Cooperative Agreement

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.

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.

Publications:

Cavazzana AO, Miser JS, Jefferson J, Triche TJ. Experimental evidence for a neural origin of Ewing's sarcoma of bone. Am J Pathol 1987;127:507-18.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09138-04 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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 20892

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, RNA transcripts and N-myc protein as detected by in situ hybridization with radiolabelled DNA fragments of the N-myc gene, transcribed in vitro and hybridized to frozen sections of approximately 80 tumors provided by one of us (RS). Likewise, N-myc protein will be detected immunocytochemically by antibodies specific for oligopeptide regions of the N-myc protein product. 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.

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.

Publications:

Cohen PS, Seeger RC, Triche TJ, Israel MA. Detection of n-myc gene expression in neuroblastoma tumors by *in situ* hybridization. Am J Pathol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09140-04 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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 20892

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.

PHS 6040 (Rev. 1/84)

GPO 914-918

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.

In addition, a polyclonal antiserum has been prepared against the 500 K protein after preparative SDS-PAGE and excision of the band, in acrylamide, from the gel. This antiserum is currently being evaluated.

Publications:

Scarpa S, Reddy MP, Mims S, Triche TJ. A novel, 500,000 molecular weight, apparent extracellular matrix protein. Exp Cell Res (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09160-02 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

N-myc Expression in Small Round Cell Tumors of Childhood

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

COOPERATING UNITS *(if any)*

C.P. Reynolds, D.J. Slamon, and R.E. Seeger, UCLA

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	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 space provided.)*

N-myc oncogene expression is an important factor in neuroblastoma prognosis and biologic behavior. N-myc has been sporadically reported in rare other childhood tumors. Further antibody detection methods have recently become available. We have used a rabbit polyclonal antibody to detect the N-myc protein in tissue sections of the common tumors of childhood. We have further correlated these results with conventional Northern, Southern, and Western analysis of examples of each of these tumors. We find no evidence of N-myc amplification, expression, or protein accumulation in any of these tumors save neuroblastoma.

This project is now complete.

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

Major Findings:

1. N-myc protein is readily detected with a rabbit polyclonal antibody and avidin-biotin immunoperoxidase in frozen sections.

2. Intensity of staining of protein by immunocytochemistry correlates well with mRNA expression by Northern analysis, even with no N-myc amplification by Southern analysis.

3. Western analysis of total protein extracts exactly corroborates the Northern and immunocytochemistry results.

4. Only some neuroblastomas express N-myc in our series; Ewing's sarcoma, peripheral neuroepithelioma, rhabdomyosarcoma, and lymphoma do not.

Publications:

Triche TJ, Cavazzana AO, Navarro S, Reynolds CP, Slamon DJ, Seeger RE. N-myc protein expression in small round cell tumors. In: Evans AE, D'Angio GJ, Knudson AG, Seeger RC, eds. Advances in Neuroblastoma Research 2. New York: Alan R. Liss, 1988;475-85.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09162-02 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

c-myc Oncogene Expression in Rhabdomyosarcoma in Relation to Tumor Growth

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

PI:	M. Tsokos	Expert	LP NCI
OTHER:	G. Kouraklis	Visiting Fellow	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 20892

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 had previously found that cells from 6 human rhabdomyosarcoma (RMS) cell lines when injected in the subcutis of nude mice retain their initial cytology (embryonal vs. alveolar). Moreover, we found correlation between the histologic subtype (alveolar vs. embryonal) of RMS and the time/size of tumor growth in the subcutis of nude mice. In addition, all cell lines were found to express high levels of the c-myc oncogene, with or without c-myc amplification. N-myc was not expressed or amplified in any of the lines.

In the present study, using the same RMS cell lines as in the previous study, we evaluated levels of expression of the c-myc oncogene in relation to (1) the histologic subtype, (2) tumor cell growth in vitro, and in the subcutis of nude mice, and (3) the ability of the tumor cells to form pulmonary nodules after tail vein injection mice.

c-myc expression was evaluated by Northern blot analysis, and densitometric analysis was performed with an ultrascan XL laser densitometer. To determine if myc expression is independent of cell proliferation, we performed Northern blot analysis of the same cells after serum deprivation.

In vitro tumor growth was evaluated by counting cells in a Coulti counter every 3 days for a total period of 12 days. In order to correlate c-myc expression with tumor growth, the results of tumor growth in the subcutis of nude mice from the previous study were used. The potential of tumor cells to cause pulmonary nodules ("metastases") was evaluated with tail vein injections of 2.5×10^5 cells from each RMS cell line into six mice per line. All mice were sacrificed after six weeks and autopsied. Pulmonary nodules were counted macroscopically and confirmed histologically.

Major Findings:

1. The levels of c-myc expression correlated with the histologic subtypes of RMS cell lines: greater (12 fold) in the alveolar than in the embryonal RMS cell lines.

2. The levels of c-myc expression were independent of cell proliferation, because the same results were obtained from serum supplemented, as well as from serum deprived cells.

3. Cell growth *in vitro* did not correlate with levels of expression of c-myc.

4. The levels of c-myc expression correlated well with tumor growth in the subcutis of nude mice: cell lines with high c-myc RNA levels caused tumor nodules that grew faster and to a greater diameter than those of the cell lines with lower c-myc RNA levels.

5. The levels of c-myc expression correlated with the ability of the RMS cells to form tumor nodules in the lungs of the nude mice: all 3 alveolar RMS cell lines with high c-myc RNA levels resulted in lung nodules proven histologically to be tumors. None of the embryonal RMS cell lines resulted in lung nodules.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00879-05 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Molecular Characterization of Hybridoma Antibodies of V_H J606 Family

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

PI: K.R. Schroer Senior Surgeon LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

Antibodies of the J606 murine V_H family, GAC (group A-streptococcal carbohydrate) (GAC-related V_H genes), have been seen to derive in normal mice from at least $3V_H$ gene segments (of a locus whose complexity is 10-12). Two of these are GAC-related, one inulin related. In CBA/N mice, which do not respond to either GAC or inulin by conventional immunization methods, antibodies of the J606 V_H family origin were found to constitute about 5% of their B cell V_H pre-immune repertoire. The extent of expression of the members of the J606 V_H family has been examined by nucleotide sequencing of hybridoma derived V_H J606 genes from their purified mRNA.

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

Major Findings:

1. Antibody MK2 (clone 245.46.3) is derived from germline V_H J606, DSp2.2, and Jh_3 segments by a V → DJ recombination which produces an anomalous join that is characterized by a 6 nucleotide deletion of the 3' end of the V_H gene. Mechanisms for such an unusual join include the possibility of unregulated excessive exonuclease activity as has been suggested for the defects of SCID (severe combined immunodeficiency) mice.

2. The MK2 clone uniquely amongst 10 V_H J606 bearing antibodies bears the anomalous join. No repetition of the anomaly has been seen in 10 additional joins of near-identical V_H sequences. The defective joining, therefore, is clearly not common in the CBA/N strain among J606 V_H genes. Furthermore, since the additional sequences are normally joined to downstream elements, the germline V_H segment undoubtedly bears the correct 3' flanking recognition sequences (heptamer/spacer/nanomer).

3. A new V_H gene family has been found to be expressed in the CBA/N strain. This gene was the source of hybridoma mRNA sequences from 2 independently derived B cell clones from a single mouse. The genetic complexity of the new V_H locus and its expression in CBA/N and other mouse strains are subjects of continuing interest.

4. Molecular rearrangements of the J606 V_H locus are found in at least two V-loci, involving an assortment of D segments and all Jh segments, apparently randomly. The genetic V_H allelic equivalent of the HGAC39 locus is not rearranged in any of the observed hybridomas. Vhgac39 is highly homologous to the expressed V_H-coding segments, but differs in its 5' flanking region markedly, suggesting that 5'regulatory elements may be important determinants of the rearrangement of this gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00559-06 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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 & Metastases Section	LP NCI
OTHER:	U. Wewer	Visiting Fellow	LP NCI
	C.N. Rao	Visiting Associate	LP NCI
	I.M.K. Margulies	Biologist	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 20892

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.)*

The overall goal of the research has been to identify and characterize biochemical mechanisms involved in the interaction of metastatic tumor cells with the extracellular matrix. A major objective has been to analyze the events which take place at the interface between the tumor cell and the basement membrane during attachment.

Accomplishments

1. Laminin, a glycoprotein of basement membranes, was shown to play a major role in metastatic tumor cell attachment.
2. The cell surface laminin receptor was purified and partially sequenced.
3. Monoclonal antibodies and polyclonal antibodies were prepared against the human breast cancer laminin receptor. These antibodies have provided new information about the distribution and function of the receptor.
4. The binding domain on the receptor and the ligand were identified. Additional functional domains were located on the ligand and a model of the attachment interface was developed.
5. A fragment of the laminin ligand was produced which blocks the receptor and markedly inhibits or abolishes hematogenous metastases in a nontoxic fashion in animal models.
6. A fragment of laminin which binds to the cell surface receptor was iodinated and found to localize preferentially to lung metastases in a murine model.
7. Antibodies to the laminin receptor were coupled to the surface of adriamycin containing liposomes. The antibody liposomes preferentially transferred adriamycin to high laminin receptor human breast carcinoma cells.

Major Findings:

1. The complete amino acid sequence of the putative binding domain (131 amino acids) on the laminin receptor for laminin has been determined based on a cDNA clone.

2. Synthetic peptides corresponding to overlapping regions in this sequence have been purified. The synthetic peptides have been coupled to glass beads and studied for their laminin binding properties. A 20 mer (met 18 pro) was identified which binds laminin with an IC50 (competition and displacement) of 10 nM and which fails to bind type I collagen, fibronectin, GRGP or GRGE.

3. The synthetic peptide corresponding to the receptor binding domain for laminin inhibits the binding of labeled laminin to cells and inhibits the haptotaxis of melanoma cells on substrate coated with a gradient of laminin.

4. Fragments of laminin and synthetic peptides corresponding to the receptor inhibit the growth of established human breast carcinoma micrometastases in nude mice.

5. Laminin binding to its receptor in isolated plasma membranes stimulates GTPase activity significantly and this is blocked by the anti-laminin receptor antibody. The receptor itself is not a G protein but cooperates with G protein transducer systems, probably through the IP3 pathway.

6. Antibodies made against synthetic peptides derived from the laminin receptor recognize the natural receptor, inhibit cell attachment to endogenous or exogenous laminin, and block laminin mediated haptotaxis.

7. Antibodies to the laminin receptor coupled to the surface of adriamycin containing liposomes result in preferential killing of high laminin receptor MDA breast carcinoma cells compared to low laminin receptor human breast epithelium.

Publications:

Grigioni WF, E'Errico A, Biagini G, Villanacci V, Mazziotti A, Liotta LA, Garbisa S, Mancini AM. Primary liver cell carcinoma. New insight for a more correct approach to its classification. Acta Path Jpn 1987;37:929-40.

Haberern-Blood C, Liotta LA, Rao CN, Kupchik HZ. Laminin expression by human pancreatic carcinoma cells in the nude mouse and in culture. J Natl Cancer Inst 1987;79:891-8.

Wewer UM, Taraboletti G, Sobel ME, Albrechtsen R, Liotta LA. Laminin receptor: Role in tumor cell migration. Cancer Res 1987;47:5691-8.

Lapis K, Paku S, Liotta LA. Endothelialization of embolized tumor cells during metastasis formation. Clin Exp Metast 1988;6:73-89.

Publications (Cont'd):

Kalebic T, Williams JE, Talmadge JE, Kao-Shan C-S, Kravitz B, Locklear K, Siegal GP, Liotta LA, Sobel ME, Steeg PS. A novel method for selection by invasive tumor cells: derivation and characterization of highly metastatic K1735 melanoma cell lines based on in vitro and in vivo invasive capacity. Clin Exp Metast (in press)

Roberts DD, Wewer UM, Liotta LA, Ginsburg V. Laminin-dependent and laminin-independent adhesion of human melanoma cells to sulfatides. Cancer Res (in press)

Guirguis R, Margulies IMK, Taraboletti G, Schiffmann E, Liotta LA. Cytokine-induced pseudopodial protrusion is coupled to tumour cell migration. Nature 1987;329:261-3.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00891-05 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Stimulated Motility 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
	L. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
OTHER:	M.L. Stracke	Biotechnology Fellow	LP NCI
	E.C. Kohn	Medical Staff Fellow	MB NCI
	R. Guirguis	Guest Researcher	LP NCI
	H. Krutzsch	Expert	LP NCI

COOPERATING UNITS *(if any)*

M.E. Lippman, Medicine Branch, NCI; C. Freter, Medicine Branch, NCI; D. Salomon, DCBD, NCI; B. Sloane, Dept. of Pharmacology, Wayne State Univ., Detroit, MI

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.1	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.)*

We have continued our studies on the biochemical properties of the motility of malignant tumor cells in as much as migration is a requirement for their metastasis. The preparation of an autocrine motility factor (AMF) from A2058 human melanoma cells has been scaled up using successive separation procedures with hydrophobic interaction and HPLC anion exchange chromatography. Studies on the mechanism of action of AMF have indicated that it may stimulate motility by activating the phosphatidyl inositol (PI) pathway. AMF enhanced both migration and PI turnover in a parallel, concentration-dependent manner. AMF also stimulated release of arachidonic acid in melanoma cells. The peptide mellitin increased adherence of cells as well as stimulating phospholipase A2 activity. AMF was also found to increase Ca^{++} fluxes in cells at early times. These results collectively implicate Ca^{++} mobilization and lipid metabolic pathways as signal processing components of the motile response in tumor cells. We have also demonstrated that insulin-like growth factors stimulate migration in melanoma cells and accomplish this via a pertussis-toxin insensitive pathway. This is in contrast to the mechanism of action of AMF which is toxin-sensitive. The significance of this may be that malignant tumor cells have alternate ways of mounting a motile response to diverse chemical stimuli, a property which could facilitate their metastatic dissemination in the host. Cell lines other than melanoma, such as ovarian carcinoma, breast and bladder cancer, and a rodent line, were shown to produce AMFs. Some of these cell types and A2058 melanoma cells respond to each other's AMF, suggesting that there may be homologous active site domains within AMFs in general. The definitive determination of this point must await sequencing AMF, a result most likely to come when the AMF gene is cloned.

GPO 914-918

Major Findings:

a) Preparation and purification of AMF

We have scaled up and improved our method for isolating AMF. Melanoma cells are grown in a 'cell factory' with medium to which has been added insulin (10 μg/ml), the protease inhibitor aprotinin (1 μM), and bovine serum albumin (5 μg/ml). Each growth cycle yields ~ 2 liters of conditioned medium. This is concentrated in an Amicon filtration cartridge containing a filter which retains material with Mr $\geq$ 30 KD. The concentrate is washed with buffered saline to remove materials with Mr < 30 KD. The final material is then applied to a phenyl sepharose hydrophobic interaction column using a reversed gradient of ammonium sulfate and a linear gradient of ethylene glycol. The column fractions were freed of ammonium salts and ethylene glycol prior to bioassay for their ability to stimulate migration. The active fractions were then applied to an HPLC quaternary anion exchange column. Biologically active fractions were collected. We are at present processing large volumes (50 to 100 liters) of conditioned medium using these procedures.

b) Mechanism of action of AMF

Our previous studies demonstrated that AMF stimulated motility in melanoma by a pertussis toxin-sensitive pathway that was independent of adenyl cyclase. We, therefore, investigated the roles of two metabolic pathways for lipids thought to be involved in motility and known to be sensitive to pertussis toxin in other systems. In melanoma cells prelabelled with ^{3}H-inositol, it was found that AMF stimulated turnover in the phosphatidyl inositol (PI) pathway. The total inositol phosphate production was increased as much as 3-fold over control with a similar increase for inositol 1,4,5 tris phosphate, the putative intracellular Ca^{++} mobilizing agent. It was also observed that AMF stimulated a coordinate, concentration-dependent increase in both motility and phosphatidyl inositol turnover, reinforcing the conclusion that this pathway plays a direct role in motility. Furthermore, pertussis toxin inhibited the PI turnover by 50 percent, further supporting our inference that stimulated motility occurs through a receptor-mediated process requiring a G-protein.

We studied the activation of phospholipase A2 (PLA) in presence of AMF in melanoma cells prelabelled with ^{14}C-arachidonic acid. Two peaks of arachidonate release over control were observed: a small one over the interval 15 to 30 minutes after adding AMF, and a large one, as much as two-fold, at about 150 minutes. The ionophore, A23187, caused a single rise in arachidonate release within 20 minutes. Agents which have been shown to affect PLA in other systems, quercetin, NDGA, ETYA, all inhibited motility to a minor extent at relatively high concentrations. The PLA-stimulating peptide mellitin, enhanced arachidonate release in melanoma cells and markedly stimulated adherence without affecting motility.

Since the PI and PLA pathways have been shown to be related to Ca^{++} mobilization in other cell types, we examined Ca^{++} fluxes in melanoma cells. Using Quin 2-loaded cells, it was found that AMF stimulated a rapid increase in intracellular Ca^{++} within minutes. This was confirmed with ^{45} Ca studies. Another increase in intracellular Ca^{++} occurred after a 2nd addition of AMF, suggesting

that the cells could adapt to changes in concentration of a motility stimulant. These results were consistent with the occurrence of both influx and intracellu lar mobilizations of Ca^{++}. Partial inhibition of motility fluxes was observed in the presence of the high Ca^{++} threshhold channel blockers nixedipine, diltiazem, and verapamil, as well as with the low threshhold blocking agent amiloride. Collectively, these findings indicate possible roles for Ca^{++} fluxes and pathways of lipid metabolism in early and later events, respectively, in the transduction of motility signals in malignant tumor cells.

c) Other motility factors

It has been found that insulin-like growth factors and insulin stimulate migration of melanoma cells. Insulin-like growth factor-I (IGF-I) is the most potent, producing a maximal response at 10 nM, while both insulin and IGF-II caused peak cell migration over a range of 300-400 nM. With ^{125}I-labelled IGF-I, it was determined by Scatchard analysis that the cells possessed a high affinity receptor (Kd ~ 3×10^{-10} M) and about 5×10^3 receptors per cell. The responses to IGF-I and insulin were principally chemotactic in contrast to that of AMF which was previously shown to be chemokinetic. However, the stimulated motility responses induced by all three insulin peptides were not inhibited by pertussis toxin, whereas the AMF effect was virtually eliminated in the presence of the toxin. These results indicate that a highly malignant cell may have a heterogeneity of systems for generating a motile response to diverse cytokines. This heterogeneity would appear to be present at both the levels of ligand-receptor interaction and signal transduction reactions. Such features may contribute in a major way to the metastatic dissemination of the cells.

In pursuit of these findings, studies have been initiated on the stimulation of motility by insulin peptides in tumor cell lines other than melanoma. These include ovarian, breast, and bladder carcinoma cells as well as the 5R rodent cell line.

Preliminary results indicate that tumor cell lines derived from epithelial neoplasms respond preferentially to IGF-II over a concentration range similar to that tested for IGF-I in melanoma cells.

d) AMF from other tumor cell lines

An ovarian tumor cell line, OVCAR-3, derived from a human tumor, was shown to migrate in response to its own conditioned medium. These cells responded both chemotactically and chemokinetically to their concentrated conditioned media. The AMF in the media was partially characterized. It is stable to heating at 56° but not at 100°, suggesting that the activity resides in a protein. The activity is stable at pH 1 but is largely inactivated at pH 11. Studies of its stability to action of proteases is underway. The OVCAR-3 cells also respond to the melanoma AMF and laminin, while the OVCAR AMF stimulates migration in A2058 melanoma cells, T-24 bladder cancer cells, and MDA 231 breast cancer cells. These results may indicate that AMFs from a variety of tumor cells may have homologous active sites with which they interact at a common cell surface receptor.

Publications:

Stracke ML, Guirguis R, Liotta LA, Schiffmann E. Pertussis toxin inhibits stimulated motility independently of the adenylate cyclase pathway in human melanoma cells. Biochem Biophys Res Commun 1987;146:339-5.

Liotta LA, Wewer U, Rao NC, Schiffmann E, Stracke M, Guirguis R, Thorgeirsson U, Muschel R, Sobel M. Biochemical mechanisms of tumor invasion and metastasis. Anticancer Drug Design 1987;2:195-2.

Liotta LA, Guirguis R, Stracke M. Biology of melanoma invasion and metastasis. Pigment Cell Res 1987;1:5-15.

Vasanthakumar G, Manjunath R, Mukherjee AB, Warabi H, Schiffmann E. Inhibition of phagocyte chemotaxis by uteroglobin, an inhibitor of blastocyst rejection. Biochem Pharmacol 1988;37:389-4.

Liotta LA, Schiffmann E. Tumor autocrine motility factors. In: DeVita VT Jr, Hellman S, Rosenberg SA, eds. Important advances in oncology 1988. Philadelphia: J B Lippincott Co, 1988;17-30.

Stracke ML, Kohn E, Taraboletti G, Liotta LA. Tumor invasion and metastasis: Biochemical mechanisms. In: Lippman M, Dickson R, eds. Breast cancer: cellular and molecular biology. Boston: Martinus Nijhoff (in press)

Stracke ML, Kohn EC, Aznavoorian SA, Wilson LL, Salomon D, Krutzsch HC, Liotta LA, Schiffmann E. Insulin-like growth factors stimulate chemotaxis in human melanoma cells. Biochem Biophys Res Commun (in press)

Guirguis R, Margulies IMK, Taraboletti G, Schiffmann E, Liotta LA. Cytokine-induced pseudopodial protrusion is coupled to tumour cell migration. Nature 1987;329:261-3.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00892-05 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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:	P.S. Steeg	Biotechnology Fellow	LP NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
	G. Bevilacqua	Guest Researcher	LP NCI
	H.C. Krutzsch	Expert	LP NCI
	C.R. King	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.8	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.)*

The molecular biology of tumor metastasis has been investigated. In previous years, a novel gene, NM23, was identified by differential colony hybridization, using RNA from related, low and high metastatic potential melanoma cell lines as probes. NM23 RNA levels have been determined in four metastasis model systems. In each case, non- or low metastatic tumor cells contained higher NM23 RNA levels than did related, high metastatic tumor cells. The NM23 gene represents the first nonimmunologically related tumor cell gene that is associated with low metastatic potential.

NM23 RNA levels were determined in human infiltrating ductal breast carcinomas, and compared to histopathological indicators and 1-2 year clinical course to evaluate the diagnostic potential of this gene. NM23 RNA levels were uniformly low in tumors from patients with 4 or more positive axillary lymph nodes at surgery. Of the patients with 0-3 positive lymph nodes at surgery, tumor NM23 RNA levels varied: Approximately 70% of the tumors contained high NM23 RNA levels, consistent with low metastatic potential. Of the patients in this group, none has experienced cancer progression. However, 30% of the 0-3 positive lymph node patients had low NM23 RNA levels; one of these patients has experienced a recurrent cancer. The NM23 gene may therefore provide a valuable supplement to current diagnostic practices.

The murine NM23 gene encodes two proteins, of 17 and 19 KDa. Rabbit antisera to NM23 peptides are being characterized, with the eventual goal of investigating NM23 protein levels in human cancers. In ongoing experiments, a cDNA clone thought to encode the 17 KDa protein has been transfected into high metastatic murine K1735 TK melanoma cells. The transfected cell lines will be tested for their metastatic potential, to determine the therapeutic potential of the NM23 gene.

Major Findings:

A novel gene, NM23, was identified by differential colony hybridization using RNA from related low and high metastatic potential murine melanoma cell lines as probes. NM23 RNA levels have been determined in four metastasis model systems: (1) murine K1735 melanoma cell lines, in which the gene was identified; (2) rat nitrosomethylurea-induced mammary tumors; (3) mouse mammary tumors induced by two strains of mouse mammary tumor virus; (4) rat embryo fibroblasts transfected with c-Ha-ras or cotransfected with ras + adenovirus 2 E1a. In each case, non- or low metastatic tumor cells contained higher NM23 RNA levels than did related, high metastatic tumor cells. The NM23 gene represents the first non-immunologically related tumor cell gene identified which is associated with low metastatic potential. It is a candidate for a gene that suppresses the malignant phenotype.

Diagnostic potential of the NM23 gene for breast cancer. Twenty-five infiltrating ductal breast carcinomas were screened for NM23 RNA levels using in situ and Northern blot hybridizations; these data were compared to traditional histopathological indicators and one-to-two year clinical course to evaluate the diagnostic potential of the NM23 gene. All tumors from patients with four or more positive lymph nodes at surgery, an indicator of an aggressive tumor, had low NM23 RNA levels. Of the patients with 0-3 positive lymph nodes, NM23 RNA levels varied: Approximately 70% of the tumors contained high NM23 RNA levels, consistent with low metastatic potential. However, 30% of the tumors from this group contained low NM23 RNA levels, equivalent to those of the four or greater positive lymph node group. Of the tumors from the 0-3 positive lymph node group with low NM23 RNA content, all were negative for at least one hormone receptor, and one patient in this group has developed recurrent cancer. None of the patients with high NM23 RNA levels has developed a disease recurrence. The NM23 gene has therefore predicted clinical course in one case where lymph node data did not. This gene may provide a valuable supplement to existing histopathological techniques to identify patients in need of more aggressive treatment in advance of disease progression.

Gene structure. A murine pNM23-1 cDNA clone was identified and sequenced. This clone contained a 700 bp cDNA insert, approximately 70% of the 1.0 Kb message. The DNA sequence and predicted amino acid sequence of the only open reading frame were novel when compared to Genebank and Protein Database sequences, respectively. Hybrid selection experiments with the pNM23-1 cDNA insert indicated two gene products, at 17 and 19 KDa. As the pNM23-1 cDNA clone contains a methionine with an optimal surrounding translation initiation sequence at a distance from a double termination codon to encode a 17 KDa protein, it is thought that the pNM23-1 cDNA clone encodes a full-length NM23 gene product. It is not known whether the 19 KDa product represents a more 5' translation initiation site along the same gene, or alternatively is a related NM23 protein.

Due to the potential diagnostic significance of the NM23 gene for human cancer, further investigation into its structure has focused on human cDNA clones. A human Okayama-Berg cDNA library has been screened, and human pNM23 cDNA clones are being characterized.

Characterization of NM23 proteins. Two peptides, corresponding to the N-terminal and most hydrophilic portions of the predicted 17 KDa NM23 protein, have been synthesized and are being used to develop antisera to NM23 protein(s). Such antisera will be used to confirm the physical characteristics of the NM23 gene product(s), and determine if NM23 proteins are differentially expressed between low and high metastatic tumor cells. If so, a simple immunohistochemical reaction could replace in situ hybridizations for diagnosis of human tumor metastatic potential. Our preliminary data indicate that antibody directed to the hydrophilic sequence immunoprecipitates a 17 KDa protein from translations of murine melanoma RNA.

Transfection of the NM23 gene. The pNM23-1 cDNA clone, which contains a SV40 promoter, has been transfected into murine K1735 TK melanoma cells, which are of high metastatic potential. The transfected cell lines will be screened for NM23 RNA levels. Any lines with significantly higher NM23 RNA content than control, neomycin resistance gene-transfected TK lines will be tested for their metastatic potential in mice, as well as for function in a variety of in vitro correlates of metastatic potential.

Publications:

Kalebic T, Williams JE, Talmadge JE, Chien-Song KS, Kravitz B, Locklear K, Siegal GP, Liotta LA, Sobel ME, Steeg PS. A novel method for selection of invasive tumor cells: derivation and characterization of highly metastatic K1735 melanoma cell lines based on in vitro and in vivo invasive capacity. Clin Exp Metast (in press)

Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA, Sobel ME. Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst 1988;80:200-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00893-05 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI
OTHER:	H. Krutzsch	Expert	LP NCI

COOPERATING UNITS *(if any)*

R. Pozzatti (Fellow), LMV, NCI; S. Garbisa, Padova Institute of Histology, Italy

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

Activated ras oncogene transfection into suitable recipient cells has been shown to induce the metastatic phenotype (Thorgeirsson et al., Mol. Cell Biol. 5: 259-262, 1985). With R. Pozzatti and G. Khoury, we extended this work to ras^H transformed rat embryo cells. These cells were also highly metastatic when transformed either by ras^H or by ras^H linked to an enhancer. However, when the cooperating oncogene Ela was used in conjunction with ras^H, metastasis was only rarely seen. We have used this model system to study the correlation of basement membrane collagenolysis with metastatic propensity. The c-Ha-ras oncogene alone, or combined with v-myc, transfected into early passage rat embryo fibroblasts, induce these cells to secrete high levels of type IV collagenolytic metalloproteinase and to concomitantly exhibit a high incidence of spontaneous metastases in nude mice. Cotransfection of c-Ha-ras plus the adenovirus type 2 Ela gene yields cells which are highly tumorigenic but nonmetastatic and fail to produce type IV collagenase. This effect is due to a suppression of collagenase elaboration, not increased production of a collagenase inhibitor, and not decreased production of a collagenase activator. Western immunoblots demonstrate a ten-fold or greater increase in the elaboration of latent type IV collagenase following ras transfection and suppression by Ela. These data support a biochemical linkage of type IV collagenase expression with the metastatic phenotype in this rodent system.

GPO 914-918

Major Findings:

We have found that the ras^H oncogene (either the viral oncogene from the Harvey sarcoma virus or the T24 bladder carcinoma oncogene) when used to transform NIH-3T3 cells results in both tumorigenic and metastatic cells. In contrast, v-sarc, v-mos transformants, or NIH-3T3 cells transformed by increased expression of normal P21 (using an LTR inserted upstream from the $c\text{-}ras^H$ gene to boost its expression, Chang et al., Nature 307: 658, 1982), are highly tumorigenic but not metastatic. This finding confirms in this system that tumorigenicity and metastatic capacity are distinct and that the ras^H oncogene has the ability to induce metastasis.

Ras^H transformed diploid cells created by Spandidos and Wilkie (Nature 3110: 469, 1984) were also metastatic as were ras^H transformed rat embryo cells. This indicated that the induction of metastatic capacity by ras^H did not depend on NIH-3T3 cells as the recipient but was a more generalizable phenomenon. Ras^H plus adenovirus Ela were not metastatic. Thus, ras induces metastases by a pathway independent of its effects on tumorigenicity.

C127 cells, on the other hand, do not become metastatic after transformation by $v\text{-}ras^H$. We showed that even if the P21 levels were increased 5-10 fold, the cells did not become metastatic. We were able to transfer DNA from a metastatic cell into these cells and select metastases in nude mice. A second transfer resulted again in production of metastatic cells. These results suggest that a second gene is required to interact with the ras^H gene and that it can be identified in gene transfer experiments.

Publications:

Liotta LA. Overview of the biology of cancer invasion and metastases. In: Rosenberg S, ed. Surgical treatment of metastatic cancer. Philadelphia: J B Lippincott (in press)

Zucker S, Turpeenniemi-Hujanen T, Ramamurthy N, Wieman JM, Lysik RM, Gorevic P, Liotta LA, Golub LM. Purification and characterization of a variant collagenase from the cytosol of metastatic melanoma cells. J Biol Chem (in press)

Liotta LA. The biology of metastases. In: Kelly WM, ed. Textbook of internal medicine. Philadelphia: J B Lippincott (in press)

Liotta LA, Wewer U, Rao CN, Schiffmann E, Stracke M, Guirguis R, Thorgeirsson U, Muschel R, Sobel M. Biochemical mechanisms of tumor invasion and metastases. In: Prodi G, Liotta LA, Lollini P-L, Garbisa S, Gorini S, Hellmann K, eds. Cancer metastasis - Biological and biochemical mechanisms and clinical aspects. New York: Plenum Press (in press)

Liotta LA, Stracke ML. Tumor invasion and metastases: Biochemical mechanisms. In: Lippman ME, ed. Growth factors in breast cancer. Breast cancer: Cellular and molecular biology. (in press)

Liotta LA. Biochemical mechanisms of tumor invasion and metastases. Clin Physiol Biochem 1987;5:190-9.

Hubbard SM, Liotta LA. The biology of metastases. Cancer Nursing: A Comprehensive Textbook. (in press)

Liotta LA. H-ras and the metastatic phenotype. (Editorial) J Natl Cancer Inst 1988;80:468.

Muschel R, Liotta LA. Role of oncogenes in metastases. Carcinogenesis 1988; 9:705-0.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09130-04 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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:	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
	C.N. Rao	Visiting Associate	LP NCI

COOPERATING UNITS *(if any)*

A. Schwartz, George Washington University Hospital, Washington, D.C.;
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 20892

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.

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

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. Using the recently devised indirect radioimmune assay, we have assayed greater than forty individual cases of breast carcinoma, infiltrating ductal carcinoma. Results from this preliminary sampling show a 6-10 fold variation in bound antibody per mg of protein assayed. We have accumulated clinical data on each case including estrogen and progesterone receptor content, clinical stage including lymph node (i.e. metastasis) status, breast quadrant location of primary tumor and in most cases classification as pre- or postmenopausal. Each tumor sample is being examined independently for histological grade and percentage of carcinoma versus other tissue elements. Analysis of histological grade, clinical status and assayed amount of laminin receptor per mg of tissue indicates a need for a more sensitive method of detection of laminin receptor content. Therefore, we have begun a screening of several antibodies raised against laminin, its receptor and fragments of both of these proteins. Using indirect immunoperoxidase methods, we have developed excellent specific staining for the laminin receptor and the ligand. Qualitative and assessment of the staining intensity as a means to determine relative amounts of the protein in various breast carcinoma tissue(s), revealed intense staining for the laminin receptor in invading breast carcinoma cells. A laminin receptor content of low value correlates with a generally accepted, less well-differentiated tumor having estrogen receptor and progesterone receptor negativity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09131-04 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters 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:	A.P. Claysmith	Biologist	LP NCI
	V. Castronovo	Guest Researcher	LP NCI
	A. Mackay	Visiting Fellow	LP NCI
	M. Fernandez	Guest Researcher	LP NCI
	D. Combs	Stay-in-School	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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 standerd 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, their regulation, and how they interact with the tumor cell, we have undertaken to construct, isolate, and characterize molecular clones of laminin receptor. The laminin receptor is a cell surface protein to which laminin (a major component of basement membrane) specifically binds with high affinity. We previously isolated a human laminin receptor cDNA which encoded the carboxy-terminal half of the protein, and showed that laminin receptor mRNA in the tumor cell is a rate-limiting control step in the biosynthesis of the receptor, and hence in the regulation of cellular attachment to basement membranes via laminin. We have also cloned the murine laminin receptor. More than one laminin receptor gene has been detected in both human and murine cells. During the past year, we have extended our cloning and sequence analysis of the laminin receptor gene in both human and mouse. We have obtained full-length murine laminin receptor cDNAs and have predicted the entire primary structure of the human and murine laminin receptor. The nascent laminin receptor is smaller than expected, suggesting that the receptor is a highly substituted molecule. Antibodies to synthetic peptides deduced from the cDNA clones have been used to study the biological role of the laminin receptor in cell migration. The expression of laminin receptor in breast cancer cells is modulated by estrogen and progesterone, consistent with the observation that the course of metastatic breast cancer is modulated by hormones.

GPO 914-918

Major Findings:

Complete sequence of human laminin receptor mRNA. We previously isolated laminin receptor cDNA clones from a human endothelial λgt11 expression library. One of the original human laminin receptor cDNA clone, λELR4, encoded 253 amino acids and contained a 69 base long 3' untranslated region. A subsequent clone, λELR14, was isolated and sequenced. It encoded an additional 38 2/3 amino acids amino terminal to λELR4. A specific synthetic oligonucleotide derived from a sequence at the 5' end of λELR14 was used as a primer to reverse transcribe mRNA extracted from human Panc-1 cells. The primer extended product was 86 bases longer than the 5' end of the cDNA insert of λELR14. The sequence of the primer extended product was 86% homologous with the 5' end of a full-length murine laminin receptor cDNA, and encoded an additional 3 1/3 amino acids as well as 75 bases of 5' untranslated sequence. The full-length human laminin receptor mRNA, excluding the poly(A) tail, is therefore 954 bases long. The complete human laminin receptor protein sequence was determined to be 295 amino acid residues long. Computer analysis of this predicted protein sequence shows several hydrophobic regions, including a short (16 residues) putative intramembrane domain. No traditional signal peptide sequence was found.

Complete sequence of murine laminin receptor mRNA. Several murine laminin receptor cDNA clones were completely sequenced. One clone, pMLR21, is a full-length cDNA, as proven by primer extension experiments. The murine laminin receptor mRNA has a 79 base long 5' untranslated region, which is 86% homologous with the human 5' mRNA. It begins with an unusual sequence of $CT_{(6)}$. The first methionine codon is not surrounded by a translation consensus sequence. Another met codon, positioned 9 residues further downstream, also does not have a typical consensus sequence. Evidence that the first met is, in fact, the initiating codon can be inferred from human sequence data, in which an out of reading frame met codon is present between met 1 and met 10. The correct reading frame was firmly established by comparing cDNA-predicted amino acid sequence and authentic laminin receptor peptide sequence data, as well as by studies showing a biological role for cDNA-predicted synthetic peptides. Antibodies to a synthetic peptide derived from met 1 to residue 9 are currently being developed to definitively prove that met 1 is, in fact, the initiating codon. The coding sequence of the murine clone is highly homologous to the human, with only 4 out of 295 amino acids differing. The nucleic acid sequences of the coding regions of the murine and human clone are 88% homologous to each other. Most changes are in wobble positions, often involving a T to C switch. This suggests that the two species use different tRNAs preferentially when translating the laminin receptor mRNA. The 3' untranslated region of the murine sequence, however, differs significantly from, and is 53 bases longer than, the human sequence.

Polymorphisms of the laminin receptor. Using genomic DNA hybridization blots, we had previously predicted that there are multiple laminin receptor genes in the human and mouse genomes. We reported the isolation of cDNA clones which cross-hybridize with authentic human laminin receptor cDNA. These clones, λELR106 and λELR112, extend several hundred bases more 5' than λELR14, which is inconsistent with the primer extension experiments described above. We have completed sequencing these clones. In the 954 bases of over-

Major Findings (Cont'd):

lapping sequence with λELR4/14, λELR106/112 both had (the same) 25 base changes, one of which would result in a stop codon approximately midway in the sequence. Interestingly, λELR106/112 sequences immediately 5' of the predicted 5' end of authentic human (and murine) laminin receptor mRNA diverge sharply from ech other and contain multiple stop codons in all reading frames. These clones presumably encode pseudolaminin receptor mRNAs which may be the result of improper splicing of intron sequences in the genome. The role that these pseudo mRNAs play in the biology of the cell is not clear. We are initiating a study of polymorphisms of the laminin receptor gene. Probes specific for the 5' ends of λELR106/112 will be hybridized to genomic DNA and compared to the pattern of hybridization to authentic λELR14/4. In addition, synthetic oligonucleotides specific to λELR106/112 vs. λELR14/4 in the coding region of the laminin receptor mRNA will be used as probes on Northern blots to determine the relative abundance of specific mRNA sequences. The pattern in normal vs. neoplastic cells will be assessed. We have also detected a single base change between λELR4 and λELR14 which would result in a change in amino acid. Other cDNA clones, λELR1, 2, and 5, which, like λELR4, were isolated by antibody screening, were completely sequenced. Two of these, λELR1/5, contain the same base change as does λELR14, but no other changes. λELR2 does not include this region. The relative abundance of λELR4 vs. λELR1/5/14 mRNAs will be determined by hybridization of RNA blots to specific oligonucleotides, as described above. Furthermore, we have isolated 98 independent plaques from a human genomic DNA phage library. These phages will be analyzed for specific "polymorphic" sequences. Use of synthetic oligonucleotides specific for authentic vs. pseudogenes will assist us in the timely identification of the regulatory regions of these genes. Sequence analysis of several murine laminin receptor cDNA clones reveals three specific sites of base changes amongst the clones which appear in varying combinations with each other. This suggests that polymorphisms exist in the murine system as well as the human.

Translation of the laminin receptor mRNA. The predicted size of laminin receptor from the cDNA coding sequence is 33 KDa. This is in sharp contrast to an apparent M_r ~ 68KDa of authentic laminin receptor on reduced SDS-polyacrylamide gels. Selective hybridization-translation experiments reveal that the human and murine laminin receptor clones select an mRNA whose translated product runs on gels with an apparent M_r ~ 38 KDa. This in vitro synthesized polypeptide is specifically recognized by the same antibody that recognizes a 68 KDa band on immunoblots of tissue and cell extracts. Apparently, the receptor is a highly substituted molecule. Although no N-linked carbohydrate sites are evident from the cDNA sequence, the receptor is rich in serine. We are currently determining O-linked oligosaccharides in the receptor, as well as other possible post-translational modifications. Our existing antibodies to the receptor do not efficiently immunoprecipitate the protein from solution. We are currently developing antibodies to synthetic peptides of the receptor as well as to recombinant laminin receptor to develop tools that can be used in biosynthetic pulse-chase experiments to better understand the post-translational processing of the laminin receptor.

Major Findings (Cont'd):

The selective hybridization translation experiments were also used in a quantitative assay to measure the translatability of laminin receptor mRNA. The content of in vitro translatable mRNA in several cell lines was correlated with the differential ability of those cells to bind to laminin, as well as to Northern blot hybridization data. These experiments showed that while multiple laminin receptor mRNAs may be present in the cell, at least a significant proportion of these mRNAs are translated in the cell. Furthermore, the ability of a tumor cell to bind to laminin appears to be controlled by the amount of laminin receptor mRNA in the cytoplasm.

Biological role of laminin receptor. To study the potential role of laminin receptor in tumor cell migration, antibodies were developed to synthetic peptides predicted by the human laminin receptor cDNA. Regions with high probability of reverse turns were selected since such structures are often involved in receptor-ligand binding domains. Antibodies directed against peptides from the carboxy-terminal end of the receptor were used in immunocytochemical studies; the data suggest that the carboxy-terminal half of the receptor is located on the exterior of the cell membrane. We are currently developing antisera to amino-terminal predicted peptides to localize the rest of the receptor. Antibodies to carboxy-domains specifically inhibit haptotaxis of tumor cells on laminin, indicating a specific role for the laminin receptor in laminin-mediated migration. These studies also confirm our earlier data indicating that the ligand-binding domain of the receptor is encompassed in the carboxy-terminal half of the protein.

Regulated expression of laminin receptor. The regulation of laminin receptor expression in tumor cells was studied in breast carcinoma and melanoma cell lines. Treatment of the metastatic breast carcinoma cell line T47D with progesterone resulted in a marked increase in laminin receptor mRNA, as well as an enhanced ability of those cells to bind to laminin. Estrogen also had a positive effect, as did treatment with a combination of estrogen and progesterone. Estrogen had no effect on MDA-MB231 cells, which have no estrogen receptors. These studies demonstrate that the expression of laminin receptor in breast carcinoma cells is modulated by hormones, consistent with the differential effect of these agents on the course of metastatic breast cancer. Growth of both breast carcinoma and melanoma cells on laminin increases the content of laminin receptor mRNA, suggesting that ligand-binding may play a positive feedback role in the production of laminin receptor message in tumor cells. Future studies will study the cellular role of laminin receptor by transfecting the complete cDNA sequence into nonmetastatic breast carcinoma cells with low receptor content.

Production of recombinant substrates. We are pursuing the large scale production of recombinant laminin receptor in prokaryotic systems. We can obtain microgram quantities of laminin receptor fusion protein from mini-cultures of the λgtll system. We are recloning the complete cDNA into other expression vectors to obtain nonfusion protein. As an adjunct to the laminin receptor studies, we are also cloning into expression vectors those portions of laminin cDNA which encode domains involved in binding to the receptor. The availability

Major Findings (Cont'd):

of recombinant ligand and receptor will enable us to study the binding process in biochemical detail.

Publications:

Wewer, UM, Taraboletti G, Sobel ME, Albrechtsen R, Liotta LA. Role of laminin receptor in tumor cell migration. Cancer Res 1987;47:5691-8.

Young MF, Shimokawa HS, Sobel ME, Termine JD. A characterization of amelogenin messenger RNA in the bovine tooth germ. Adv Dent Res 1987;1:289-2.

Shimokawa, H, Ogata Y, Sasaki S, Sobel ME, McQuillan CI, Termine JD, Young MF. Molecular cloning of bovine amelogenin cDNA. Adv Dent Res 1987;1:293-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09159-02 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Thrombospondin and its Receptors: Role in Cell Adhesion and Motility

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

PI:	G. Taraboletti	Visiting Fellow	LP NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
	S. Aznavoorian	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

D. Roberts, Laboratory of Structural Biology, NIDDKD, NIH

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

Thrombospondin induces the migration of human melanoma and carcinoma cells. Using a modified Boyden chamber assay, tumor cells migrated to a gradient of either soluble thrombospondin (chemotaxis) or substratum bound thrombospondin (haptotaxis). A series of human melanoma and carcinoma cells exhibited different levels of response when compared for their relative motility stimulation by thrombospondin haptotaxis versus chemotaxis. Human A2058 melanoma cells which exhibit a strong haptotaxic and chemotaxic response to thrombospondin were used to study the structural domains of thrombospondin required for the response. Using fragments of thrombospondin and monoclonal antibodies to the COOH and NH_2 domains, it was found that the COOH domain mediated haptotaxis and the NH_2-domain mediated chemotaxis. Pertussis toxin treatment inhibited chemotaxis but not haptotaxis implicating separate G protein transducer systems for each type of motility.

Major Findings:

Thrombospondin stimulated the migration of human melanoma cells in the modified Boyden chamber assay, in a dose dependent manner. An increase in migration occurred within a range of 1.0 to 150 nM and plateaued at thrombospondin concentrations higher than this. The checkerboard analysis, in which the migration of tumor cells is studied using different ratios of thrombospondin above and below the filter, indicated that migration was directional in nature, occurring to the highest level in a positive gradient (maximum of 27 fold). Tumor cells migrated over substratum bound thrombospondin in the absence of soluble thrombospondin. The migration of cells to a substratum bound attractant, termed haptotaxis, was pronounced in the presence of a step density gradient of substratum bound thrombospondin with a plateau above 60 nM.

A series of human melanoma and breast carcinoma cell lines were compared for their migration response to a soluble or solid phase gradient of thrombospondin. Six separate human tumor cell lines exhibited wide differences in their relative migration induced by thrombospondin.

To determine which regions of the thrombospondin molecule are responsible for chemotaxis and haptotaxis, three approaches were used:

1) Affinity purified antibodies recognizing specific domains of thrombospondin were studied for their ability to inhibit thrombospondin mediated haptotaxis or chemotaxis. A striking difference in the antibody specificity was noted. Monoclonal antibody C6.7, which recognizes the globular carboxyl terminus of thrombospondin, inhibited haptotaxis in a dose-dependent manner, with a maximum of inhibition (90%) at the concentration of 10 μg/ml. In contrast to its inhibition of haptotaxis, antibody C6.7 had no significant dose dependent effect on thrombospondin induced chemotaxis. Monoclonal antibody A2.5, which recognizes the amino terminus of thrombospondin, exhibited the opposite pattern for haptotaxis versus chemotaxis inhibition when compared to antibody C6.7. Antibody A2.5 inhibited thrombospondin chemotaxis in a dose dependent manner, but had no significant dose dependent effect on haptotaxis. Antibody A6.1, which recognizes the core 70 kDa internal domain fragment of thrombospondin, did not significantly inhibit haptotaxis and only slightly inhibited chemotaxis.

2) Roberts et al. have reported that fucoidan, heparin and the monoclonal antibody A2.5 which bind to the amino terminal domain of TSP, selectively inhibit melanoma cell spreading, but only weakly inhibit attachment. Therefore, the inhibitory effects of heparin and fucoidan were studied for thrombospondin mediated tumor cell migration. Heparin and fucoidan both showed a dose dependent inhibition of thrombospondin chemotaxis but had no effect on, or in some cases slightly stimulated, haptotaxis. This finding is in keeping with a major role for the amino terminal domain in the mediation of chemotaxis but not haptotaxis.

3) Thrombin digestion of thrombospondin in the presence of different concentrations of Ca^{++} followed by further separation on a heparin affinity column yields two classes of fragments. The 140 kDa fragment contains the COOH

terminal end of thrombospondin but lacks the heparin binding domain residing in a region located at the NH2 terminal domain of intact thrombospondin. The 140 kDa fragment can be further cleaved to a 120 kDa fragment which lacks the COOH binding region for Mab C6.7. Both the 120 and the 140 kDa fragments have identical amino terminal sequences. The thrombospondin fragments were studied for the ability to promote tumor cell migration. The 140 and the 120 kDa fragments were both devoid of chemotactic activity. In contrast, the 140 kDa fragment retained full ability to elicit haptotaxis with a response equal to or greater than the control undigested thrombospondin. When the 140 kDa fragment was partially cleaved to the 120 kDa form, significant haptotaxis was lost. Furthermore, the fully cleaved 120 kDa fragment was unable to generate a haptotaxis response. The 120 kDa fragment lacks the C6.7 binding site which is retained on the 140 kDa fragment.

The thrombospondin fragment results are consistent with the antibody and thrombospondin binding molecules inhibition data, further demonstrating that the heparin binding amino terminal region of thrombospondin is required for chemotaxis, whereas the C6.7 binding COOH terminal region of thrombospondin is required for haptotaxis.

Treatment of A2058 melanoma cells with pertussis toxin (PT) reduced thrombospondin mediated chemotaxis in a dose dependent manner. However, PT did not alter haptotaxis. This provides evidence that separate G proteins are involved in haptotaxis versus chemotaxis.

Publications:

Taraboletti G, Roberts DD, Liotta LA. Thrombospondin-induced tumor cell migration: Haptotaxis and chemotaxis are mediated by different molecular domains. J Cell Biol 1987;105:2409-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09161-02 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Laminin Receptor and its Role in the Function of Natural Killer Cells

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

PI:	G.J. Bryant	Expert	LP NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI

COOPERATING UNITS *(if any)*

J. Hiserodt, Pittsburgh Cancer Institute, Pittsburgh, PA

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

Natural killer (NK) cells, a subpopulation of large granular lymphocytes (LGL) are involved in the spontaneous lysing of tumor or virally infected target cells. The killing action requires binding of the killer cells to the target cells. The role of the laminin receptor in this process of specific binding is being examined in our laboratories. Using murine NK cells, the presence of laminin-like molecule and a receptor for laminin binding has been established (Kd 10^{-11} M, approximately 990,000 receptors/cell).

Further studies will attempt to define and compare the receptor on NK cells versus the already established laminin receptor located on many tumor cells; the interactions between these two receptors during cell lysis will be explored.

GPO 914-918

Major Findings:

Results from live cell binding assays using radiolabeled laminin indicate a relative increase of specific binding for murine NK cells over LGL and thymocytes. Immunocytochemical studies have demonstrated binding of the polyclonal antibody raised against the laminin receptor to the cell surface of NK, LGL and thymocytes; though the qualitative intensity was less for the latter two cell types.

Publications:

Bryant GJ, Rao CN, Brentani M, Martins W, Lopes JD, Martin SE, Liotta LA, Schiffmann E. A role for the laminin receptor in leukocyte chemotaxis. J Leukocyte Biol 1987;41:220-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09163-01 LP

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Anticancer Effects of a Novel Drug, Merck L651582

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

PI:	E.C. Kohn	Medical Staff Fellow	MB NCI
OTHER:	E. Schiffmann	Research Chemist	LP NCI
	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI

COOPERATING UNITS *(if any)*

C. Meyers, Chief, Med. & Clin. Pharmacol. Branch, NCI; C. Freter, Medicine Branch, NCI; D. Hupe, Merck Research Labs., Rahway, NJ

LAB/BRANCH
Laboratory of Pathology

SECTION
Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	1.1	0.5

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.)*

In collaboration with Merck, Rahway, NJ, we are evaluating a coccidiostatic drug produced by that company for its antiproliferative and antimetastatic effects. Merck has previously shown that the drug inhibits indirectly several steps in nucleotide biosynthesis that are required for cell proliferation in a number of mammalian cell types. This compound was also found to inhibit the production of inositol trisphosphate, an agent that mobilizes intracellular Ca^{++} in both kidney cells and leukocytes. In leukocytes stimulated with a chemoattractant, L651582 reduced the influx of Ca^{++} but did not affect the development of the rapid internal Ca^{++} signal. Ca^{++} is generally required for activation of the cytoskeleton during mitogenesis. In our studies, L651582 was shown to inhibit growth, motility, and adhesion in human tumor cells and transformed rat embryo cells. In melanoma cells the drug inhibited phosphatidyl inositol (PI) turnover by 45%. Preliminary in vivo studies indicated that this agent reduced the size and number of pulmonary metastases that developed in mice after subcutaneous inoculation of tumor cells. An effect was also observed in the reduction of the number of pulmonary metastases in mice that had been injected via the tail vein with tumor cells pretreated with the drug. It is planned to pursue these studies on metastasis, to determine the effect of the drug upon longevity in tumor-bearing mice, and to compare these results with those produced by known chemotherapeutic agents in animal models. Development of a phase I/II clinical protocol for the drug is planned in collaboration with the Medicine/Clinical Pharmacology Branch, NCI.

GPO 914-918

Major Findings:

A) Antimetastatic properties: L651582 inhibited AMF-stimulated motility of melanoma, ovarian, breast, and rat embryo fibroblast transformants at 1 mcg/ml. This dose also inhibited monocyte migration stimulated by a formyl peptide. At this dose, it also inhibited adhesion to tissue culture plastic. Cells pretreated with L651582 were assayed for their biochemical response to AMF. A2058 melanoma cells were cultured with L651582 for 24 hours and then, after the drug was removed, were labelled for PI turnover assay. Phosphatidyl inositol turnover was inhibited up to 50% compared to untreated AMF control cells. Metastasis studies in nude mice have been performed. Significantly fewer pulmonary metastases were documented in mice receiving cells treated overnight with L651582 prior to intravenous injection. C57bl/6j mice were subcutaneously injected with PMT fibrosarcoma and on day 6 began receiving oral dosing with this drug. At doses of 100-500 mg/kg/day, a 10 to 100 fold decrease was seen in the number of spontaneous pulmonary metastases observed. Further studies are planned to evaluate L651582 effect against OVCAR3 which grows as malignant ascites and also metastasizes.

B) Antiproliferative effects: Clonogenic assays for cell replication were performed using two independent systems: ^{3}H thymidine, and crystal violet nuclear staining. Human melanoma, breast, ovarian, and bladder carcinoma cell lines were inhibited by as much as 60, 60, 60, and 95% respectively after incubation with L651582. The most striking results were generally seen at 5-10 mcg/ml. In vivo studies are planned to address efficacy and longevity in mice inoculated with melanoma, breast, and ovarian cells.

C) Clinical application: We feel that this agent represents a new class of antitumor agents. It is cytostatic, arresting the cells prior to their entry into S phase. The mechanism of action appears to be at or near the interaction of a G protein with a receptor and/or effector enzyme. Minimal toxicity has been demonstrated in laboratory rats and dogs in safety studies done at Merck. Specifically, no major toxicities were seen in the following systems: bone marrow, liver, gastrointestinal, and renal. In vitro studies suggest that this drug has anti-inflammatory effects, and we have shown that it inhibits leukocyte motility which can be necessary for host defenses against certain infections. A Phase I clinical protocol is currently being developed in collaboration with the Medicine Branch, NCI. We plan to begin with either oral or intravenous dosing to clarify the pharmacokinetics of the drug and to address efficacy in this setting.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09164-01 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Role of Collagenolytic Metalloproteinases in Metastases

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

PI:	W.G. Stetler-Stevenson	Senior Staff Fellow	LP NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
	H.C. Krutzsch	Expert	LP NCI
	M. Wacher	Visiting Scientist	LP NCI
	A. Levy	Microbiologist	LP NCI

COOPERATING UNITS *(if any)*

K. Tryggvason, Oulu, Finland; S. Garbisa, Padova Institute of Histology, Italy

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

In order to investigate the molecular properties of the type IV collagen metalloproteinase, we have obtained primary sequence information and using this data, have developed monospecific antibodies. The sequence information identifies type IV collagen metalloproteinase as a unique enzyme with some homology to other extracellular matrix degrading metalloproteinases. Synthetic peptides generated using this sequence information were used to make anti-type IV collagenolytic metalloproteinase antibodies. These antibodies were used to investigate the mechanism of activation of the collagenase and to screen an expression library to obtain a cDNA clone.

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

Major Findings:

Type IV collagenolytic metalloproteinase is a neutral metalloproteinase that degrades type IV collagen in a specific fashion and is associated with the metastatic phenotype. This enzyme is secreted in a latent form as a 70 kD polypeptide chain by human A2058 melanoma cells in culture. In vitro assays of enzyme activity have required preactivation with trypsin. In studies employing ras oncogene transfected rat embryo fibroblasts and NIH 3T3 cells, the secretion of type IV collagenolytic activity has been linked by biochemical enzymatic assay to the metastatic phenotype following transformation. In order to study the molecular properties and mechanism of activation of this enzyme, we have obtained both primary structure data and monospecific polyclonal antibodies to the latent form of human type IV collagenolytic metalloproteinase. The enzyme was purified from the culture media of human A2058 melanoma cells using standard methods. This material was sequenced directly to obtain sequence information on the amino terminus. In addition, this material was used to generate cyanogen bromide and trypsin digests from which peptide fragments were purified by reverse phase HPLC and subsequently sequenced. This sequence information identified type IV collagenolytic metalloproteinase as a unique member of a family of metalloproteinases that degrade extracellular matrix constituents and includes interstitial collagenase and stromelysin.

This sequence information was used to generate synthetic peptides corresponding to the amino-terminus of the enzyme as well as an internal cyanogen bromide fragment. Polyclonal antibodies were developed using these peptides as antigens. These antibodies were shown to be monospecific by competition ELISA experiments. Both antibodies recognized a single species migrating at 70 kD on Western blotting and immunoprecipitated the type IV collagenase from A2058 conditioned media. Electrophoresis of these immunoprecipitates on gelatin zymograms demonstrated the association of gelatin degrading activity with the latent type IV collagenase.

In vitro studies of the mechanism of type IV collagenase activation were conducted using p-aminophenylmercuric acetate (pAPMA), a compound which has been shown to activate the interstitial collagenase in vitro. Incubation of type IV collagenolytic metalloproteinase-containing conditioned media from human A2058 melanoma cells with pAPMA resulted in gradual conversion of the single gelatin degrading band of activity associated with the type IV collagenase to a doublet with the upper band migrating in its original position. With continued incubation, this doublet is slowly converted to a triplet with the appearance of a new, lower molecular weight species as seen on gelatin zymograms. This data suggests that activation of latent type IV collagenase requires two steps and a reduction in the molecular mass. Western blot experiments using both the antibody to the amino-terminal region, COL4AT, and to the internal cyanogen bromide fragment peptides, CB4, have confirmed the loss of the amino-terminal fragment of type IV collagenase upon in vitro activation with the organomercurial pAPMA.

Attempts to obtain a cDNA clone for the type IV collagenolytic metalloproteinase have included screening lambda gtll A2058 cDNA library with synthetic oligonucleotide mixtures based on the limited protein sequence available, screening an Okayama-Berg type A2058 cDNA library with a synthetic oligonucleotide based

Major Findings (Cont'd):

on the metal-ion-binding region of human fibroblast collagenase and human stromelysin, and screening a lambda gt11A2058 cDNA library by induction of fusion protein synthesis and using the antibodies COL4AT and CB4 to immunoscreen. The attempts using synthetic oligonucleotides have been unsuccessful. However, screening the expression library has yielded twelve clones, eight which reacted with the COL4AT antibody and four which reacted with the CB4 antibody. Further characterization of these clones is in progress.

Publications:

Hoyhtya M, Turpeenniemi-Hujanen T, Stetler-Stevenson W, Krutzsch H, Tryggvason K and Liotta L. Monoclonal antibodies to type IV collagenase recognize a protein containing limited sequence homology with interstitial collagenase stromelysin. FEBS Lett (in press)

Liotta LA, Stetler-Stevenson WG. Principles of molecular cell biology of cancer: cancer metastasis. In: DeVita VT, Jr., Hellman, S, Rosenberg SA (eds.): Cancer: principles and practice of oncology, 3rd ed. Philadelphia: J B Lippincott Co. (in press)

Liotta LA, Wewer U, Rao CN, Schiffmann E, Stracke M, Guirguis R, Thorgeirsson U, Muschel R, Sobel M. Biochemical mechanisms of tumor invasion and metastases. Anti-Cancer Drug Design 1987;2:195-2.

Liotta LA, Stracke ML, Wewer UM. Tumor invasion and metastases: Biochemical mechanisms. In: Magrath I, ed. New directions in cancer treatment. Springer-Verlag (in press)

Liotta LA, Stracke ML, Wewer UM, Schiffmann E. Tumor invasion and metastases: Biochemical mechanisms. In: The pathobiology of neoplasia. Plenum Press (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00550-08 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Immunologic Characterization of Malignant Lymphomas

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:	J. Cossman	Senior Investigator	LP NCI
	D.L. Longo	Senior Investigator	MB NCI
	L.M. Neckers	Research Chemist	LP NCI
	M.A. Bookman	Senior Investigator	MB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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 standard 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. Morphologic features are analyzed to achieve improved classification of lymphoproliferative lesions.

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.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

A clinicopathologic study of patients with angiocentric immunoproliferative lesions was concluded. The concept of the angiocentric immunoproliferative lesions was developed as a unifying approach for lymphoid proliferations previously classified as lymphomatoid granulomatosis, polymorphic reticulosis, and some types of malignant lymphoma. The angiocentric immunoproliferative lesions (AIL) represent a distinct clinicopathologic type of lymphoproliferative disease. The lesions all have an angiocentric and angiodestructive pattern of growth. A histologic grading scheme was proposed which describes three histologic grades. Grade 1 lesions have little or no cytologic atypia and a polymorphous inflammatory background. Grade 2 lesions demonstrate moderate cytologic atypia but still retain a polymorphous inflammatory background. Grade 3 lesions show marked cytologic atypia and lack an inflammatory background and are further designated as angiocentric lymphomas. Immunophenotypic studies demonstrated a mature T-cell phenotype in all cases studied. One case classified as angiocentric lymphoma demonstrated a clonal rearrangement of the T-cell receptor beta gene. Patients with grade 1 and grade 2 lesions had been initially treated with cyclophosphamide and prednisone. Progression to malignant lymphoma occurred in 3 of 9 patients with grade 1 disease and 4 of 6 patients with grade 2. Supervening lymphomas were usually refractory to subsequent aggressive therapy, with only one patient achieving a complete remission. In contrast, 7 of 8 patients with grade 3 lesions achieved complete remission with aggressive combination chemotherapy.

These studies support the concept that the angiocentric immunoproliferative lesions represent a spectrum of post-thymic T-cell proliferation. The single most important prognostic indicator for ultimate survival is achieval of an initial complete remission. Aggressive combination chemotherapy may be necessary to achieve complete remission in patients with grade 2 and 3 lesions. Patients treated initially with conservative chemotherapy may be compromised in their ability to achieve a complete remission if they recur or progress to a more high grade lesion.

Nodular lymphocyte predominant Hodgkin's disease had recently been suggested to be a B-cell derivation based on phenotypic and morphologic findings. A compilation of 7 unique cases of NLPHD was described. In these cases lymphohistiocytic mononuclear variant cells (LNH) became clustered into increasingly large aggregates. In areas of the same tumor mass, large confluent sheets of these cells resembled large cell lymphoma. However, in contrast to what would be expected for large cell lymphoma, all patients had localized disease clinically, and 6 of 7 achieved long-term disease-free survival following radiation therapy and/or chemotherapy (in 2 cases) for Hodgkin's disease. No patient developed disseminated large cell lymphoma. Also notable was a high frequency of axillary lymph node involvement (5 of 7) and occurrence in individuals of the black race (6 of 7). Immunophenotypic and molecular genetic analyses were suggestive of a B-cell derivation for the proliferating cells in those cases studied. These findings support a B-cell derivation for lymphocyte predominant Hodgkin's disease and indicate that histologic progression to a process resembling large cell lymphoma may occur.

Major Findings (Cont'd):

An immunohistochemical study of the distribution of the c-myc protein was completed. By using various fixation procedures on cytospin preparations of HL60 cells, we found the subcellular distribution of the c-myc protein to be dependent upon the method of fixation. In a study of mouse tissues in frozen sections using a biotinylated monoclonal antibody, we found the protein to be widely distributed in various normal adult mouse tissues, in most cases localized to the nucleus. When the same tissues were studied after formalin fixation and paraffin embedding, there was a loss of nulcear staining concurrent with the appearance of c-myc protein immunoreactivity in the cytoplasm. It was concluded that immunohistochemical studies of the expression of this oncogene should take into consideration the effects of fixation when examining its subcellular distribution.

Publications:

Ulirsch RC, Jaffe ES. Sjögren's syndrome-like illness associated with the acquired immunodeficiency syndrome-related complex. Hum Pathol 1987;18: 1063-8.

Lipford EH, Smith HR, Pittaluga S, Jaffe ES, Steinberg AD, Cossman J. Clonality of angioimmunoblastic lymphadenopathy and implications for its evolution to malignant lymphoma. J Clin Invest 1987;79:637-2.

Jaffe ES, Bookman MA, Longo DL. Lymphocytic lymphoma of intermediate differentiation - mantle zone lymphoma: A distinct subtype of B-cell lymphoma. Hum Pathol 1987;18:877-0.

Jaffe ES. Malignant lymphomas: Pathology of hepatic involvement. Semin Liver Dis 1987;7:257-68.

Cohen PJ, Jaffe ES. Current methods used in the diagnosis and classification of malignant lymphomas. In: DeVita VT Jr, Hellman S, Rosenberg SA, eds. Cancer: principles & practice of oncology, 2nd ed. Updates, Vol. 1, No. 8. Philadelphia: J B Lippincott Co., 1987;1-12.

Jaffe ES, Lipford EH Jr, Margolick JB, Longo DL, Cossman J, Fauci AS. Lymphomatoid granulomatosis and angiocentric lymphoma: A spectrum of post-thymic T-cell proliferations. Sem Resp Med (in press)

Loke S-L, Neckers LM, Schwab G, Jaffe ES. c-myc protein in normal tissue: Effects of fixation on its apparent subcellular distribution. Am J Pathol 1988;131:29-7.

Jaffe ES. An approach to the classification of post-thymic T-cell malignancies. Proceedings of the International Colloquium on Lymphoid Malignancies, Philadelphia, Field and Wood, Inc. (in press)

Publications (Cont'd):

Sundeen JT, Cossman J, Jaffe ES. Lymphocyte predominant Hodgkin's disease nodular subtype with coexistent "large cell lymphoma": Histological progression or composite malignancy? Am J Surg Pathol (in press)

Steinberg A, Seldin M, Jaffe ES, Smith H, Klinman D, Krieg A, Cossman J. Angioimmunoblastic lymphadenopathy with dysproteinemia. Ann Intern Med (in press)

Jaffe ES. Pulmonary lymphocytic angiitis: A nosologic quandary. (Editorial) Mayo Clinic Proc 1988;63:411-3.

Solomon D, Merino MJ, Linehan M, Jaffe ES. Angiocentric malignant lymphoma involving lung and kidney: mimicking renal cell carcinoma in fine needle aspiration cytology. Surg Pathol (in press)

Cohen PJ, Jaffe ES. Histopathology and immunophenotyping. Chapter 3. In: Magrath I, ed. The non-Hodgkin's lymphomas. Edward Arnold, Publ. (in press)

Jaffe ES. Histiocytoses of lymph nodes: Biology and differential diagnosis. Semin Diag Pathol (in press)

Barriga F, Whang-Peng J, Lee E, Morrow C, Jaffe ES, Cossman J, Magrath IT. Development of a second clonally discrete Burkitt's lymphoma in a human immunodeficiency virus (HIV) positive homosexual patient. Blood (in press)

Jaffe ES. Immune markers in the diagnosis of malignant lymphomas. J Virol Meth (in press)

Colamonici OR, Cole D, Rosolen A, Kirsch I, Felix C, Poplack DG, Jaffe ES, Neckers LM. Stimulation of the beta subunit of the IL-2 receptor induces MHC-unrestricted cytotoxicity in T acute lymphoblastic leukemia cells and normal thymocytes. J Immunol (in press)

Young RC, Longo DL, Glatstein E, Ihde DC, Jaffe ES, DeVita Jr, VT. The treatment of indolent lymphomas: Watchful waiting versus aggressive combined modality treatment. Semin Hematol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00552-08 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

PI:	J. Cossman	Senior Investigator	LP NCI
OTHER:	M. Raffeld	Senior Staff Fellow	LP NCI
	J. Sundeen	Biotechnology Fellow	LP NCI
	P. Cohen	Biotechnology Fellow	LP NCI
	M.A. Stetler-Stevenson	Expert	LP NCI
	A. Bakhshi	Senior Investigator	MB NCI
	C. Hua	Guest Worker	MB NCI

COOPERATING UNITS *(if any)*

Medicine Branch, NCI; Metabolism Branch, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	.50

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 frequent relapse of follicular lymphoma, the major obstacle to cure, is a consequence of clonal expansion of daughter cells derived from a common stem cell. Thus, despite the "clinical" remission achieved by therapy in most patients, residual lymphoma cells must persist. To detect occult lymphoma, we have specifically amplified the joined bcl-2/JH DNA sequences created by the t(14;18) translocation seen in nearly all follicular lymphomas. Using multiple rounds of primer-directed DNA polymerization (polymerase chain reaction, PCR), we can detect 1 copy of bcl-2/JH, which is 4 orders of magnitude more sensitive than flow cytometry or Southern blot restriction analysis. Genomic DNA sequence analysis of four lymphomas confirmed that the size of the amplified fragment serves as a unique tumor marker. Direct application to clinical samples has demonstrated lymphoma cells which were otherwise undetectable.

Human T cell neoplasms are nearly always clonal and show coincident rearrangement of their T cell receptor beta and gamma genes. The T cell receptor delta gene is deleted from both chromosomes in most mature T cell neoplasms but is retained and frequently rearranged in precursor T and B cell neoplasms (ALL). Our findings demonstrate an ordered hierarchy whereby human delta gene assembly precedes rearrangement of either the beta or gamma genes and involves an extremely narrow pattern of V-delta usage. Many of the additional rearrangements we have observed in the delta locus may be due to chromosomal translocation in this region (14q11) which is frequently translocated in T cell neoplasms.

Major Findings:

1. Detection of a single follicular lymphoma cell has been made possible by DNA amplification.

2. The T cell receptor delta gene rearranges early, before beta and gamma genes and is rearranged in T-ALL and pre B-ALL.

Publications:

Cossman J, Uppenkamp T. T-cell gene rearrangements and the diagnosis of T-cell neoplasms. In: Davey FR, eds. Clinics in laboratory medicine, Vol. 8, No. 1. New York: W B Saunders, 1988;31-44.

Raffeld M, Wright JJ, Lipford N, Cossman J, Bakhshi A, Korsmeyer SJ. Clonal evolution of t(14;18) follicular lymphomas demonstrated by immunoglobulin genes and the 18q21 major breakpoint region. Cancer Res 1987;47:2537-2.

Cossman J, Uppenkamp M, Sundeen J, Coupland R, Raffeld M. Molecular genetics and the diagnosis of lymphoma. Arch Pathol Lab Med 1988;112:117-7.

Steinberg A, Seldin M, Jaffe ES, Smith H, Klinman D, Krieg A, Cossman J. Angioimmunoblastic lymphadenopathy with dysproteinemia. Ann Intern Med (in press)

Cossman J, Sundeen J, Uppenkamp M, Sussman E, Wahl L, Coupland R, Lipford E, Raffeld M. Rearranging antigen receptor genes in enriched Reed-Sternberg cell fractions of Hodgkin's disease. Hematolog Oncol (in press)

Lipford E, Wright JJ, Urba W, Whang-Peng J, Kirsch IL, Raffeld M, Cossman J, Longo D, Bakhshi A, Korsmeyer SJ. Refinement of lymphoma cytogenetics by the 18q21 major breakpoint. Blood 1987;70:1816-3.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00850-06 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Clonal Analysis 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	Senior Staff Fellow	LP NCI
	R. Andrade	Visiting Fellow	LP NCI
	R. Coupland	Guest Researcher	LP NCI
	D. Cohen	Senior Investigator	LCP NIDDKD

COOPERATING UNITS *(if any)*

Laboratory of Clinical Biology, NIDDKD

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.25	.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.)*

We have determined the incidence with which the t(14;18) chromosomal translocation involves each of the known DNA breakpoint clusters of the bcl-2 gene in a series of 85 cases of follicular lymphomas. Four breakpoint clusters were identified and the location of the breakpoint may influence the course of disease. Despite t(14;18) translocation, a substantial group (15%) was identified which did not rearrange bcl-2 loci in any of these breakpoint regions, indicating that additional breakpoints remain. We are attempting to clone the DNA sequences at new breakpoint regions and link them to the bcl-2 transcriptional unit.

The recently discovered gene encoding the delta chain of the second human T cell receptor lies within an actively rearranging region of the T cell receptor alpha locus. Using DNA probes of this region, we have identified T-delta gene rearrangement as a frequent, early event in precursor T-cell neoplasms (ALL). Prior to our analysis, only one delta variable (V) gene was known. In the course of our study, we have discovered two new productive V-delta genes and together the three V genes may comprise the entire delta V repertoire.

In an attempt to identify DNA sequences at the site of chromosomal translocation in Hodgkin's disease, we have cloned and sequenced the rearranged genomic DNA region of both the immunoglobulin heavy chain gene and T cell receptor beta chain gene in the Hodgkin's disease cell line, L428. The rearranged VH gene we cloned from L428 is a member of the recently discovered VH-V family reported as frequently rearranged in B cell neoplasms. However, we find VH-V either germline or deleted, but only rarely rearranged, in B cell ALL, CLL and follicular lymphoma.

Major Findings:

1. bcl-2, the translocating, putative oncogene involved in follicular lymphoma, contains more than four breakpoint regions.

2. The delta gene of the second T cell receptor contains 3 V genes and is frequently rearranged in precursor B and T cell neoplasms.

3. The Hodgkin's disease cell line, L428, has rearranged one of the recently discovered VH-V genes thought to be frequently involved in B cell neoplasms.

Publications:

Cossman J, Uppenkamp M. T-cell gene rearrangements and the diagnosis of T-cell neoplasms. In: Davey FR, ed. Clinics in laboratory medicine, Vol. 8, No. 1. New York, W B Saunders, 1988;31-44.

Bakhshi A, Wright JJ, Graninger W, Seto M, Owens J, Cossman J, Jensen JP, Goldman P, Korsmeyer SJ. Mechanism of the t(14;18) translocation: Structural analysis of both derivative 14 and 18 reciprocal partners. Proc Natl Acad Sci USA 1987;84:2396-0.

Tsujimoto Y, Bashir MM, Givol I, Cossman J, Jaffe ES, Croce CM. DNA rearrangements in human follicular lymphoma can involve the 5' or the 3' region of the bcl-2 gene. Proc Natl Acad Sci USA 1987;84:1329-1.

Uppenkamp M, Pittaluga S, Coupland R, Colamonici O, Cossman J. Stable $T3^-$ and $T3^+$ subclones derived from the mosaic human T cell leukemia cell line, CEM. J Immunol 1988;140:2802-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00855-06 LP

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pathologic Features of HTLV-I 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
	P. Levine	Senior Investigator	EEB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.05	.03	0.02

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. Prospective studies of all lymphomas in similar geographic regions with differing incidences of adult T cell leukemia/lymphomas are studies to discern factors which make an impact on the incidence of HTLV-I and HTLV-I associated diseases.

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

Major Findings:

Ninety-six patients with the diagnosis of adult T-cell leukemia lymphoma (ATL) were identified in countries outside of Japan and the Caribbean basin. Seventy-four of these patients were initially diagnosed in the United States; 25 of 52 patients whose place of birth was known, had been born in the United States. The detection of 14 patients born in the southeastern United States, all black, indicates a group deserving particular attention for studies of HTLV-I. A registry for ATL cases was established which will assist in the identification of risk factors for this disease, and at the same time improve case definitions and early diagnosis.

In a separate study, serum samples were obtained over a 2 year period from 136 Panamaian patients with hematologic malignancies. Only 3 patients had clinical and serologic findings of HTLV-I-associated ATL. It was concluded that although classical ATL does occur in the Panamanian population, it is not as prevalent as in other Caribbean populations.

Publications:

Levine PH, Jaffe ES, Manns A, Murphy EL, Clark J, Blattner WA. Human T-cell lymphotropic virus type I and adult T-cell leukemia/lymphoma outside of Japan and the Caribbean basin. Yale J Biol Med (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00881-07 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Regulation of Lymphocyte Activation and Proliferation

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

PI:	L.M. Neckers	Research Chemist	LP NCI
OTHER:	J.B. Trepel	Biologist	NMOB NCI
	R. Nordan	Staff Fellow	LG NCI
	O. Colamonici	Visiting Associate	LP NCI
	S. Loke	Visiting Fellow	LP NCI
	X. Zhang	Guest Researcher	LP NCI
	J. Cohen	Research Chemist	LCP NCI
	A. Rosolen	Guest Researcher	PB NCI

COOPERATING UNITS *(if any)*

Laboratory of Genetics, NCI
Naval Medical Oncology Branch, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	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.)*

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.

We have begun to study the role of nuclear proteins in the transition of T cells from G_0 to S phase. In an initial study, a c-myc antisense oligomer completely blocked the appearance of c-myc protein in mitogen treated T cells, yet these cells went on to express IL-2 receptors, transferrin receptors and DNA polymerase α protein. Yet they failed to synthesize DNA. We plan to continue to use antisense constructs to various nuclear proteins to study their role(s) in cell growth and activation.

We have synthesized derivatized oligos to study their stability in culture and have developed acridine labeled oligos with which we are studying oligo uptake by cells. We are also investigating the targeted delivery of oligos to cells by lipofusion, as a route to eventual clinical treatment. We have initiated antisense projects to study the role of n-myc in neuroblastomas and c-fos in proliferation and differentiation.

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.

7. Using an antisense c-myc oligomer, we have shown that induction of IL-2 receptors, transferrin receptors, DNA polymerase α and blastogenesis in mitogen treated T cells does not require c-myc protein.

8. We have shown that c-myc protein induction is not necessary for $G_0 \rightarrow G_1$, traversal, as was previously thought, but that c-myc protein is required for DNA synthesis.

9. We are the first to demonstrate the feasibility of using antisense oligomers to study the role(s) of specific nuclear proteins in cell activation and proliferation.

10. Using an in vitro DNA polymerase α assay system, we have determined that both c-myc protein and a nuclear protein termed K_i-67 are required for DNA polymerase α activity.

11. We are the first to describe the nature of the uptake mechanism of oligos by cells.

12. We have shown that, using liposomal fusion, targeted delivery of oligos to cells is possible.

13. We have demonstrated the utility of phosphorothioate oligos as oncogene inhibitors.

Publications (Cont'd):

Trepel JB, Colamonici OR, Kelly K, Schwab G, Watt RA, Sausville EA, Jaffe ES, Neckers LM. Transcriptional inactivation of c-myc and the transferrin receptor in dibutyryl cyclic AMP-treated HL-60 cells. Mol Cell Biol 1987; 7:2644-8.

Heikkila R, Schwab G, Wickstrom E, Loke SL, Pluznik DH, Watt R, Neckers LM. A c-myc antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G_0 to G_1. Nature 1987;328:445-9.

Heikkila R, Trepel JB, Cuttitta F, Neckers LM, Sausville EA. Bombesin-related peptides induce calcium mobilization in a subset of human small cell lung cancer cell lines. J Biol Chem 1987;262:16456-60.

Loke, SL, Neckers LM, Schwab G, Jaffe ES. c-myc protein in normal tissue: Effects of fixation on its apparent subcellular distribution. Am J Pathol (in press)

Neckers LM, Nordan R. Regulation of murine plasmacytoma transferrin receptor expression and G_1 traversal by plasmacytoma growth factor. J Cell Physiol (in press)

Neckers LM, Tsuda H, Weiss E, Pluznik DH. Differential expression of c-myc and the transferrin receptor in G_1 synchronized Ml myeloid leukemia cells. J Cell Physiol 1988;135:1-6.

Stein CA, Mori K, Loke SL, Subasinghe C, Shinozuka K, Cohen JS, Neckers LM. Phosphorothioate and normal oligodeoxynucleotides with 5'-linked acridine: Characterization and preliminary kinetics of cellular uptake. Gene (in press)

Loke SL, Stein CA, Zhang X, Avigan M, Cohen J, Neckers LM. Delivery of c-myc antisense phosphorothioate oligodeoxynucleotides to hematopoietic cells in culture by liposome fusion: Specific reduction in c-myc protein expression correlates with inhibition of cell growth and DNA synthesis. Curr Top Microbiol Immunol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09146-03 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Molecular Biology of Transferrin Receptor Expression

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

PI:	L.M. Neckers	Research Chemist	LP NCI
OTHER:	J. Trepel	Biologist	NMOB NCI
	L. Wolff	Senior Investigator	LG NCI
	X. Zhang	Guest Researcher	LP NCI
	S.L. Loke	Visiting Fellow	LP NCI

COOPERATING UNITS *(if any)*

Laboratory of Genetics, NCI
Navy Medical Oncology Branch, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

Although it is clear that transferrin receptors are regulated in part by intracellular iron, it is also clear that other regulating mechanisms are involved. Since probes to the transferrin receptor gene have recently become available, we are utilizing molecular biology techniques to study the regulation of transferrin receptor expression at the molecular level. One part of this project involves the study of mouse plasmacytoma mRNA. We have found that there are two transferrin receptor mRNAs in these cells. One message lacks the untranslated part of the full-length mRNA, while retaining the coding sequences for the protein.

Using this model system, we plan to study the ability of iron to regulate TfR mRNA levels at the level of the message. We will determine if the untranslated part of the message is necessary for iron regulation.

Another part of the project involves the regulation of TfR gene transcription in HL-60 cells by cAMP. We have shown that cAMP shuts off transcription of both c-myc and TfR within 30' - 2 hrs after addition. We will investigate the mechanism of this shut-off and determine if the c-myc gene has any effect on transcription of the TfR gene.

We have produced a TfR construct which contains only the coding region of the cDNA under LTR control. We have successfully transfected 3T3 cells with this construct and have demonstrated the existence of human TfR on their surface by immunoprecipitation and FACS analysis. We will use these cells to study the regulation of the virally controlled TfR sequence.

Using these TfR viruses, we have demonstrated that HL-60 differentiation as well as the differentiation of MEL cells is altered.

Major Findings:

1) All mouse plasmacytoma cells possess two mRNAs for the TfR. The smaller of the two is unique to plasmacytoma cells.

2) In HL-60 cells cAMP addition shuts off c-myc and TfR gene transcription in a sequential manner (by 30' and 2 hrs, respectively) long before cell growth arrests and differentiation is evident.

3) Human TfR minus the 3'-untranslated part of its message can be expressed as a fully assembled protein in 3T3 cells, when under LTR control.

4) HL-60 cells infected with the TfR virus are more sensitive to dbcamp induced differentiation than control cells.

5) In virally infected cells, Fe citrate alone is capable of stimulating rapid monocytoid differentiation.

6) In MEL cells, viral infection results in lowered responsiveness to the differentiating agent DMSO.

Publications:

Trepel JB, Colamonici OR, Kelly K, Schwab G, Watt RA, Sausville EA, Jaffe ES, Neckers LM. Transcriptional inactivation of c-myc and the transferrin receptor in dibutyryl cyclic AMP-treated HL-60 cells. Mol Cell Biol 1987;7:2644-8.

Katsaros D, Tortora G, Tagliaferri P, Clair T, Ally S, Neckers L, Robins RK, Cho-Chung YS. Site-selective cyclic AMP analogs provide a new approach in the control of cancer cell growth. FEBS Lett (in press)

Tortora G, Tagliaferri P, Clair T, Colamonici O, Neckers LM, Robins RK, Cho-Chung YS. Site-selective cAMP analogs at micromolar concentrations induce growth arrest and differentiation of acute promyelocytic, chronic myelocytic, and acute lymphocytic human leukemia cell lines. Blood 1988;71:230-3.

Tagliaferri P, Katsaros D, Clair T, Neckers LM, Robins RK, Cho-Chung YS. Reverse transformation of Ha-MuSV-transformed NIH/3T3 cells by site-selective cAMP analogs. J Biol Chem (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09147-03 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Defective TfR in HTLV-I Infected Human T Cells

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

PI:	L.M. Neckers	Research Chemist	LP NCI
OTHER:	C.A. Vidal-Soto	Guest Researcher	LP NCI
	O.R. Colamonici	Visiting Associate	LP NCI
	S. Matsushiya	Fogarty Fellow	COP NCI
	H. Mitsuya	Fogarty Fellow	COP NCI
	S. Broder	Senior Investigator	COP NCI

COOPERATING UNITS *(if any)*

Clinical Oncology Program, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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 standard unreduced type. Do not exceed the space provided.)*

We have found that HTLV-I infected T cells express 10-20 fold more TfR on their surface, but that this is due to a redistribution of the receptor from cytoplasm to surface and not to an increased receptor synthesis. Further, the TfR in these cells is poorly internalized and poorly phosphorylated by phorbol ester. The receptor cannot deliver iron to these cells, which nevertheless require iron for proliferation. Antibodies which prevent iron binding to the TfR still inhibit the growth of these cells.

GPO 914-918

Major Findings:

1) HTLV-I infected T cells express 10-20 fold more TfR on their surface than control cells.

2) This is due to receptor redistribution, not synthesis.

3) These receptors are poorly internalized and phosphorylated.

4) The TfR gene in infected cells appears to be rearranged.

Publications:

Vidal C, Matsushita S, Colamonici O, Trepel J, Mitsuya H, Broder S, Neckers LM. Altered surface expression of the transferrin receptor following HTLV-I infection *in vitro*. J Immunol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09149-03 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Differentiation of Immature T Cell Neoplasms by Interleukin-2

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

PI:	L.M. Neckers	Research Chemist	LP NCI
OTHER:	O.R. Colamonici	Visiting Associate	LP NCI
	J.B. Trepel	Biologist	NMOB NCI
	D.G. Poplack	Senior Investigator	PB NCI
	I. Kirsh	Senior Investigator	NMOB NCI
	A. Rosolen	Guest Worker	PB NCI

COOPERATING UNITS *(if any)*

NMOB, NCI; PB, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

We have studied 45 cases of T cell and myeloid leukemia trying to further characterize them. In 15 cases, these cells respond to treatment with IL-2, in that they develop the ability to kill tumor cells. Their phenotype changes to resemble that of a more mature T cell. Using these cases, we have studied the role of the T cell receptor in generation of cytotoxic activity. In one case, IL-2 induced a proliferation of cells utilizing TCR γδ. We demonstrated that this TCR was functional in that anti-CD3 stimulated an elevation of intracellular [Ca^{2+}] and augmented MHC-unrestricted cytolysis. Trigerring of the TCR γδ in this case leads directly to release of cytolytic granule enzymes.

In studying the role of IL-2 in this process, we observed that the changes we recorded following IL-2 were transduced solely via the P75 IL-2 binding protein and not the P55 (TAC) protein. Based on these findings, we have begun a clinical protocol, in collaboration with DRS, Poplack and Kirsh, to study the effectiveness of rIL-2 in vivo as a treatment for immature T cell malignancies.

In studying LGL proliferations, normal thymocytes and T-ALL cells, we have found that IL-2 augments cytolytic activity solely through stimulation of the β subunit of the IL-2 receptor. An analysis of hematopoietic neoplasms by affinity cross-linking techniques reveal that immature cells of either T, B or myeloid origin express only the β subunit of the IL-2 receptor, while more mature cells express both α and β subunits. The β subunit is capable of signal transduction in immature T cells, LGL cells, B-CLL and B-WDL cells. We are currently in the process of making a monoclonal antibody to the β subunit of the IL-2 receptor which we plan to use to screen a cDNA library made from the immunizing cells.

GPO 914-918

Major Findings:

1) We found T-cell malignancies capable of IL-2 induced MHC-unrestricted killing.

2) The phenotype analyzed by using monoclonal antibodies showed similarity among all responding cases (T/myeloid stem cell).

3) In one well-studied case, IL-2 induced utilization of TCR γδ in induction of MHC-unrestricted cytolysis.

4) TCR γδ activation leads directly to release of cytolytic granule enzymes.

5) The responding cases do not possess the P55 (TAC) IL-2 binding protein but do express the P75 IL-2 binding complex.

6) IL-2 is capable of inducing phenotypic and functional maturation of immature T cell neoplasms via the P75 IL-2 binding protein.

7) We have established a clinical protocol to study the effectiveness of rIL-2 in vivo as a treatment for some immature T cell malignancies.

8) IL-2 stimulates MHC-unrestricted killing by normal and leukemia LGL cells via stimulation of the β subunit of the IL-2 receptor.

9) The β subunit of the IL-2R, unlike the α subunit, is widely expressed throughout the hematopoietic lineage.

Publications:

Colamonici OR, Quinones R, Rosolen A, Trepel JB, Sausville E, Phares JC, Gress R, Poplack D, Weber J, Schechter GP, Neckers LM. The beta subunit of the interleukin-2 receptor mediates interleukin-2 induction of anti-CD3 re-directed cytotoxic capability in large granular lymphocytes. Blood 1988; 71:825-8.

Colamonici OR, Ang S, Quinones R, Henkart P, Heikkila R, Gress R, Felix C, Kirsch I, Longo D, Marti G, Seidman JG, Neckers LM. IL-2 dependent expansion of $CD3^+$ large granular lymphocytes expressing T cell receptor-δγ. Evidence for a functional receptor by anti-CD3 activation of cytolysis. J Immunol 1988;140:2527-3.

Colamonici OR, Cole D, Rosolen A, Kirsch I, Felix C, Poplack DG, Jaffe ES, Neckers LM. Stimulation of the beta subunit of the IL-2 receptor induces MHC-unrestricted cytotoxicity in T acute lymphoblastic leukemia cells and normal thymocytes. J Immunol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09151-03 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Immunopathology of LAK-IL2 Treated Tumors

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:	P. Cohen	Biotech. Fellow, Hematopath. Section	LP NCI
	M. Lotze	Senior Investigator, Surgery Branch	C DCT COP
	S. Rosenberg	Chief, Surgery Branch	C DCT COP
	R. Steis	Acting Chief, Clinical Res. Branch	BRMP FCRF
	J. Clark	Senior Investigator	BRMP FCRF

COOPERATING UNITS *(if any)*

Surgery Branch, DCT, NCI
BRMP, FCRF

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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

A

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

Lymphocytes activated with IL2 both in vivo and in vitro are being used for the experimental treatment of human malignancies. The mechanism of action of this therapy is not clearly established, and it is not known whether those lymphokine-activated killer cells (LAK) infused actually migrate and infiltrate tumors, or whether other effector cells mediate the observed tumor regression. In order to better understand the pathophysiology of LAK-IL2 treatment, tumor biopsy specimens are obtained before, during, and after conclusion of LAK-IL2 treatment. Specimens are studied with an immunohistochemical technique for a battery of tumor markers as well as markers capable of identifying T cells, B cells, histiocytes, NK cells, and other effector cells. These data are then correlated with conventional, clinical and pathologic data to determine whether the immunopathology observed can predict clinical response.

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

Major Findings:

A study of the immunopathology of sequential tumor biopsies in patients treated with interleukin-2 was completed and published. The study demonstrated a correlation between HLA-DR expression on the tumor cells and clinical responsiveness to IL2 and T-cell infiltration. In those patients showing an objective clinical response, a marked lymphocytic infiltrate was seen in tumor specimens. The infiltrate was composed predominantly of CD8 positive cytotoxic suppressor T lymphocytes. A significant infiltrate of NK cells or B cells was not observed. A lymphocytic infiltrate was not identified in patients failing to show a clinical response. In 4 of 5 responders, tumor cells were positive for HLA-DR before therapy and in the remaining responder, the tumor cells became positive during treatment. Tumor cells and all biopsy specimens from non-responders were DR-negative before and after the start of therapy. It was concluded that the expression of HLA-DR by tumor cells may play a role in the response to IL2 and that marked infiltration by T cells accompanies, and possibly mediates such a response.

Publications:

Cohen PJ, Lotze MT, Roberts JR, Rosenberg SA, Jaffe ES. The immunopathology of sequential tumor biopsies in patients treated with interleukin-2. Correlation of response with T-cell infiltration and HLA-DR expression. Am J Pathol 1987;129:208-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09144-04 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Identification of Proteins Binding to c-myc Regulatory Sequences

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

PI:	D.L. Levens	Acting Chief, Gene Regulation Section	LP NCI
OTHER:	M. Takimoto	Visiting Fellow	LP NCI
	M. Avigan	Medical Staff Fellow	LP NCI
	A. Farina	Guest Researcher	LP NCI
	S. Pittaluga	Visiting Associate	LP NCI

COOPERATING UNITS *(if any)*

Dr. M. Zajac-Kaye, Medicine Branch, DCBD, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.2	3.7	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.)*

Application of an exonuclease assay developed in this laboratory to identify and map the sites of tight protein-DNA interactions on large pieces of DNA, to the c-myc has revealed multiple cis- and trans-elements both upstream and downstream of the c-myc promoter P1. We have focused on 3 regions, each of which interacts with one or more sequence-specific binding proteins.

1) A binding site was discovered in c-myc intron one between Pvu II and Sac I restriction enzyme cleavage sites. Significantly, the homologous segment cloned from the abnormal c-myc allele in a Burkitt lymphoma cell line, PA682, which carries a variant t8:22 chromosomal translocation at least 16 kb downstream of the c-myc gene, possessed no binding site for this factor. Because PA682 cells still attenuate transcription at the 3' end of exon 1, it is apparent that disruption of the protein-DNA interaction at this site does not abolish attenuation. Inspection of the sequences from 6 additional Burkitt lymphomas reveals mutations within the binding site in 5 of 7 cases. The characterization of the proteins which bind to this site are in progress. It is apparent that a complex of peptides interacts within this sequence.

Insertion of a 20 bp oligonucleotide comprising the binding sequence of this protein next to a CAT (chlorampenicol acetyl transferase) gene driven by the SV40 enhancer and promoter, results in a dramatic decrease in CAT expression following transfection into mammalian cells. Thus, application of our long-range mapping procedure has resulted in the identification of a control element potentially important in the pathogenesis and progression of Burkitt's lymphoma.

2) The initiation of c-myc transcription is down regulated during DMSO induced differentiation of HL60 promonomyelocytic leukemia cells. We have identified a factor which binds to a 32 bp segment approximately 1500 bp upstream of c-myc promoter P1. This factor, or its ability to bind DNA are down regulated, with kinetics virtually superimposable on the shut-off of transcription initiation. Biochemical and genetic experiments characterizing the protein factor and better defining the exact sequence requirements for binding are in progress. A variety of transfection experiments should elucidate the function of this element in vivo.

3) A negative acting cis-element has been mapped to a 26 bp segment approximately 330 bp upstream of the c-myc promoter P1. This element has been shown to interact with at least two distinct and separable proteins. Each of these proteins interacts with sequences of viral enhancers (SV40 and gibbon ape leukemia) associated with positive effects on transcriptional initiation. To better answer if positive acting trans-factors in the context of a viral enhancer may serve as negative factors governing c-myc expression, binding sites derived from viral sequences which bind single factors have been cloned upstream from c-myc CAT gene promoter fusion plasmid constructions. These constructs are presently being used in a variety of transient and stable transfection systems to assess the physiological relevance of each factor. Preliminary analysis using specific antibodies to alter protein-DNA complex mobility using a gel retardation assay suggests a potential interaction of the c-fos protein or a fos-related antigen with one of the protein(s) which interact with this c-myc upstream negative cis-element.

Major Findings:

1. A negative cis-element is present in intron one of the c-myc gene.

    a. This element is frequently mutated in Burkitt's lymphoma.
    b. A multi-peptide complex interacts with this element.

Major Findings (Cont'd):

2. A protein regulated during the DMSO induced differentiation of HL60 cells binds far upstream (approx. 1500 bp) of c-myc promoter P1.

3. Proteins binding to the SV40 enhancer and/or the gibbon ape leukemia virus enhancer bind to a short segment approximately 330 bp upstream of c-myc promoter P1. c-fos or a fos-related antigen may be a constituent of this complex.

Publications:

Hay N, Bishop JM, Levens DL. Regulatory elements that modulate expression of human c-myc. Genes & Development 1987;1:659-71.

Zajac-Kaye M, Gelmann EP, Levens DL. A point mutation in the c-myc locus of a Burkitt's lymphoma abolishes binding of a novel nuclear protein. Science (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09168-01 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Analysis of a Multiprotein Complex Interacting with the Gibbon Ape Leukemia

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

PI:	D.L. Levens	Acting Chief, Gene Regulation Section	LP NCI
OTHER:	J. Quinn	Visiting Associate	LP NCI
	A. Farina	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.8	.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 gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with cellular proteins. These same proteins also bind to a negative cis-element approximately 330 bp upstream of c-myc promoter P1. The gibbon T-cell lymphoma cell line MLA 144 strongly transactivates the GALV-Seato enhancer. Fractionation of MLA 144 extracts by conventional and affinity chromatography allows the separation of the GALV enhancer binding protein complex into 2 components. One of these components interacts strongly with DNA but does not in itself possess full specificity. The second component does not in itself bind DNA, but upon forming a complex with the first component confers greatly enhancer power to discriminate between different sequences. The second component apparently possesses a latent DNA binding site which can be unmasked by partial proteolysis.

Contact point analysis and chemical footprinting of the GALV enhancer with the protein components described above reveals that a portion of the AP1 consensus sequence is protected by the protein which possesses reduced specificity. Upon addition of the second component, the size of chemical footprint is increased suggesting that the recognition of additional base pairs augments the sequence specificity.

PHS,6040 (Rev. 1/84)

GPO 914-918

Major Findings:

1. The 22 bp segment which is the principal determinant of GALV-Seato enhancer activity interacts with a complex of at least 2 proteins.

2. One of these proteins binds avidly but without full specificity.

3. The second of these proteins confers additional specificity, probably by allowing additional DNA protein contact.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09167-01 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Mechanisms of Interferon Regulated Gene Expression

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

PI:	A.C. Larner	Staff Fellow	LP NCI
OTHER:	H. Akai	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

I am interested in the mechanisms by which hormones and growth factors, which exert their actions through binding to cell surface receptors, are able to control the expression of specific genes. The interferons are included in this group of growth regulators and provide an attractive model to study the induction of new gene activity. An understanding of the mechanisms by which the interferons regulate the expression of certain genes should provide more insight into the pleotropic effects of these proteins.

During the past four years, I have isolated two cDNAs which correspond to two mRNAs whose expression is induced in an all or none fashion by type I interferons in a variety of human cultured cells. The induction of these mRNAs is mediated by an increase in the rate of transcription of these genes. The regulation of the transcription of the genes is complex, and involves both positive factors whose expression does not require new protein synthesis, and negative elements which inhibit the expression of these genes in certain types of cells after prolonged exposure to interferon. The action of these negative regulatory factors requires the synthesis of new proteins.

Recently, I and others have isolated and characterized the regulatory regions of interferon-sensitive genes. The availability of the cDNAs (mentioned above) and the regulatory regions of these interferon-induced genes has allowed a more detailed investigation of the expression of these mRNAs to be initiated.

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

Major Findings:

I am presently pursuing two approaches to define the mechanisms by which interferons are controlling the transcription of defined genes.

1) Attempts are being made to design functional in vitro assays to selectively transcribe an interferon-regulated gene (ISG-54K) using nuclear extracts prepared from interferon treated human fibroblasts. These studies have met with encouraging results in that nuclear extracts prepared from cells treated with interferon demonstrate a 2-5 fold increase in the transcription of ISG-54K compared with nuclear extracts prepared from untreated cells.

2) Initial studies have shown that phorbol esters inhibit the expression of the two interferon-regulated genes that I have been investigating. The effects of phorbol esters are specific, and require the synthesis of new proteins (i.e. are inhibited by treatment of cells with cycloheximide). Using transient DNA transfections, I am presently defining those DNA elements necessary for phorbol ester inhibition of the interferon-induced expression of the genes. I want to determine if the sequences involved in the action of phorbol esters are the same or different from those sequences which repress the transcription of these genes after prolonged interferon treatment. I have now mapped the regulatory sequences required for long-term incubation of cells by IFN to inhibit the transcription of genes that it initially activates. This sequence is in the same (40 base pair) region that is necessary for IFN to activate the expression of these interferon-sensitive genes. Initial correlative evidence suggests that these negative transcriptional regulatory elements may be the same. Protein synthesis is required for both the effects of phorbol esters and for prolonged interferon treatment to inhibit the expression of the genes. In addition, those cultured cells (human fibroblasts and melanoma cells) which demonstrate interferon-induced desensitization which requires protein synthesis also show phorbol ester inhibition of the interferon-sensitive genes. In cell types (Hela and HL60) which do not display an interferon-induced desensitization, phorbol esters also do not inhibit interferon induction of these mRNAs. These types of experiments will allow me to define more precisely the pathways by which interferon both activates and then represses the expression of the same set of transcription units.

Publications:

Sato T, Ye T, Larner A, Larner J. Contrasting interactions between phorbol ester and insulin on the regulation of glycogen synthase activity and p33 mRNA accumulation in rat hepatoma cells. Arch Biochem Biophys 260;1988: 377-87.

Sato T, Larner A, Larner J. The differential actions of insulin and putative insulin mediators on p33 mRNA accumulation in H4 hepatoma cells. Biochem Biophys Res Commun (in press)

Sato, Larner, A., Larner, J. Insulin-stimulated specific gene transcription in nuclear extracts from rat hepatoms cells. Endocrinology (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09169-01 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Development of Leukocyte Cell-Substrate Adhesive and Migratory Ability

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

PI: G.E. Davis, Medical Staff Fellow, LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

Leukocytes have the ability to adhere and migrate *in vitro* and *in vivo* in response to various stimuli such as inflammatory mediators. The biochemical and genetic regulation of leukocyte-substrate adhesive and migratory ability is clearly a complex process with multiple components. Such components include 1) the expression of specific cell surface receptors for leukocyte adhesion molecules and extracellular matrix molecules, 2) the expression of appropriate cytoskeletal components and their regulators and 3) the expression of additional cell surface and released components such as proteases, protease inhibitors, proteoglycans and carbohydrates. A possible advantage in attempting to study this regulation is the use of a developmental system where cells can be induced to acquire cell-substrate adhesive abilities. HL-60 promyelocytic leukemia cells are known to be deficient in cell-substrate adhesive ability unless treated with differentiating agents which can induce either a granulocyte-like or monocyte-like phenotype. I have treated HL-60 cells with various known differentiating agents such as phorbol esters (TPA), dimethylsulfoxide (DMSO), 1,25 dihydroxyvitamin D3 (vitamin D3), dibutyryl cyclic AMP and retinoic acid and have examined the development of cell-substrate adhesive and migratory ability as well as the presence of proteolytic activities. To briefly summarize, the HL-60 cells can be induced to become adhesive and migratory with individual or combinations of differentiating agents mimicking the behavior of differentiated leukocytes. In addition, the cells release a 94 kDa gelatin-degrading metalloprotease which is induced during the development of cell-substrate adhesive ability with phorbol esters.

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

Major Findings:

I have found that HL-60 cells can be induced to adhere and migrate when treated with appropriate differentiating agents. Specifically, the cells will strongly adhere to uncoated tissue culture plastic wells if treated with TPA alone (after 8-12 hours) or if first treated with DMSO or vitamin D3 for 48 hours followed by a 1-2 hour treatment with TPA. In addition, DMSO- or vitamin D3-treated cells will strongly migrate in a Boyden-chamber apparatus if exposed to appropriate levels of TPA.

A monoclonal antibody (60.3- obtained from Dr. P. Beatty, University of Washington) directed against the common beta-subunit of the leukocyte adhesion receptor family [LFA-1, MAC-1, (150,95)] blocks the cell-substrate adhesion induced by TPA alone or when used in combination with DMSO and vitamin D3. Additional monoclonal antibodies directed against leukocyte common antigen (T200) or the individual alpha subunits of the leukocyte adhesion receptors listed above do not block adhesion when used alone. However, when the alpha subunit antibodies are used in combination, blocking effects can be demonstrated suggesting that the receptors are used together in inducing adhesion. These data also demonstrate that the well described leukocyte adhesion receptors are clearly involved in the substrate adhesive interactions of differentiated HL-60 cells. Further studies will focus attention on the mechanism of action of TPA particularly in the setting where it rapidly induces the adhesive and migratory behavior of DMSO- and vitamin D3-treated cells.

When HL-60 cells are treated with TPA, they adhere to the culture substrate but also are induced to release a 94 kDa gelatin-degrading metalloprotease. This enzyme was purified using gelatin affinity chromatography and rabbit polyclonal antibodies were made. These antibodies will specifically immunosequester the 94 kDa enzyme but not a series of lower molecular weight gelatin-degrading enzymes (using gelatin-containing protease indicator gels). The antibodies also block the activity of the enzyme although preliminary experiments show that these antibodies fail to block HL-60 cell-substrate adhesion. Further experiments are underway to obtain protein sequence information for the molecular cloning of the enzyme and also to determine the distribution of the enzyme in normal and neoplastic human tissues using immunohistochemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09170-01 LP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Identification of Genes Differentially Expressed in Developing Embryonic Limb Buds

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

PI: S. Mackem Senior Staff Fellow LP·NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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 elucidation and characterization of genes necessary for establishing pattern formation during morphogenesis and the study of their regulation are problems which are central to many aspects of vertebrate biology. It may be expected that processes including responses to trophic stimuli, cell-cell interactions, migration, differential cell multiplication, programmed cell death, etc. are all involved. Many of these same processes are recapitulated in a pathologic manner during oncogenesis. Chick limb development is an attractive system for studying the molecular basis for pattern formation during morphogenesis. Critical events at the level of tissue/cellular interactions involved in pattern formation have been well characterized in this organism, the events regulating pattern formation appear to be overall very similar to those in mammalian systems, and it is possible to isolate limb bud tissue from defined stages of development in large numbers. It is the aim of this long-term project to isolate genes that regulate morphogenesis in the chick embryo limb bud.

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

Major Findings:

I have focused on approaches to identify genes that are differentially expressed during different stages of chick limb bud development using cDNA cloning and subtractive hybridization techniques. For the purpose of generating hybridization-subtraction probes, early stage (17-18) and late stage (21-22) limb bud mRNAs were extracted from about 2000 individually dissected chick embryos. These stages were chosen as likely to represent times at which signals regulating anterior-posterior and dorso-ventral patterning are just beginning to be expressed ("early"), and times at which morphogenetic gradients are well established ("late"). The isolation of differentially expressed mRNAs from these different stages should identify genes that are involved in establishing pattern formation, or at the very least, "marker" genes that are regulated by such morphogenetic signals. In addition, considering the profound differences in morphology between fore and hind limb buds in birds which is determined by the mesenchyme of the limb bud at a very early stage, wing and leg bud mRNA populations were extracted separately at the indicated stages, to allow identification of mRNAs differentially expressed in wing or leg limb buds.

Because of the relatively small amounts of mRNA that can be isolated even from a large number of limb buds (< 1 mm length each), and considering that regulatory signals may be expressed in only a small proportion of the total limb bud cell population at a given stage, techniques were sought to amplify limiting amounts of mRNA so that hybridization subtraction probes could be easily generated and to increase the likelihood of isolating low abundance sequences. The different mRNA populations were stably amplified via directional cDNA cloning into a λ bacteriophage vector (λ zap) in which the oriented cDNA insert is flanked by T3 and T7 bacteriophage promoters. The amplified cDNA population can then be transcribed to specifically yield large amounts of either sense or antisense RNA for use in the generation of hybridization subtraction probes. In addition, a modified polymerase chain reaction procedure using primers that flank the phage cDNA inserts is being developed, which would allow the generation of amplified single-stranded cDNA pools for use in subtractive hybridizations. This latter technique could be useful for selectively amplifying the single-stranded (differentially expressed) cDNA isolated during, and/or after, multiple rounds of subtractive hybridization to increase the yield of low abundance sequences.

To date, four different cDNA libraries have been constructed in λ zap using mRNA isolated from early (st 17-18) and late (st 21-22) stage wing and leg buds. Each library contains approximately 10^7 independent clones, ranging in size from 300 to 2600 bp. Modified procedures (as described above) are currently being developed and optimized for enhancing the isolation of differentially expressed, low abundance sequences from these libraries by subtractive hybridization techniques.

ANNUAL REPORT OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1987 through September 20, 1988

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 that are associated with 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 the immunoglobulin genes and T-cell antigen-specific receptor genes and the rearrangements and deletions of these genes that are involved in the control of the synthesis of immunoglobulins and T-cell receptors; one focus in this area is the study of novel transforming genes that translocate into the immunoglobulin gene locus in certain B-cell neoplasms and into the T-cell receptor locus in T-cell tumors; 2) the character- ization of transacting regulatory factors that mediate lymphocyte specific gene transcription; the scientific focus of this area is the purification of the transactivating factors, the cloning of the genes encoding these factors and the definition of their mode of action at a molecular level; 3) somatic gene therapy for human genetic immunodeficiency diseases; 4) genetic control of the immune response, especially as related to immune response genes associated with the major histocompatibility complex; one emphasis of this area is the development of a novel method for predicting molecular structures recognized by T-cells and the applications of this algorhythm to the development of vaccines aimed at preventing AIDS and other diseases; 5) identification, purification, and molecular genetic analysis of unique cell surface determinants, especially receptors for growth factors such as interleukin-2 on subpopulations of lymphoid cells with different functional capabilities using antibodies developed with hybridoma technology; 6) 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;and 7) isolation and characterization of biological response modifiers and oligosacharides 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 diseases as well as those with nonneoplastic disorders that facilitate the development of techniques for the study of cell membranes, homeostatic mechanisms, and metabolic derangements of biochemical control mechanisms are being investigated. Within this area, one special emphasis is placed on the normal growth factors especially insulin-like growth factors that play a role in the hormonal control of normal and malignant growth. An additional focus is on the regulatory role on intermediary metabolism played by proline and its metabolite pyrroline-5-carboxylate.

MOLECULAR ANALYSIS OF TRANSLOCATIONS OF TRANSFORMING GENES TO THE LOCUS OF IMMUNOGLOBULIN AND T-CELL ANTIGEN RECEPTOR GENES

Previously Dr. Thomas Waldmann and Dr. Stanley Korsmeyer introduced the analysis of rearrangements of the immunoglobulin and T-cell receptor genes to define the clonality of abnormal lymphocyte proliferations, for the classification of neoplasms of uncertain lineage, and to define the state of differentiation of neoplastic T- and B-cell precursors. Over the past year these studies have been extended to define that the abnormal lymphocytes of patients with the T-gamma lymphoproliferative disease represent a clonal proliferation of T lymphocytes. In contrast the infiltrating lymphocytes in the thyroid of patients with Hashimoto's thyroiditis or thyrotoxicosis as well as those infiltrating the intestine in patients with regional enteritis or ulcerative colitis represent polyclonal T- and B- lymphocytes.

A number of human lymphocytic tumors manifest a translocation between a transforming gene and a locus of the rearranging antigen-specific genes. Dr. Ajay Bakhshi has characterized the t(14;18)(q32;q21) chromosomal translocation present in the malignant cells in the majority of patients with follicular lymphoma and has studied the candidate transforming gene at 18q32. He has shown that the t(14;18) translocation results in the deregulated expression of the chimeric transforming gene/immunoglobulin gene (bcl-2/IgH) mRNA transcripts with somatic mutations in the bcl-2 protein coding region.

Patients with ataxia telangiectasia have a familial disorder characterized by oculocutaneous telangiectasia, cerebellar ataxia, varying degrees of immunodeficiency, an increased incidence of malignancy associated with an increased sensitivity to ionizing irradiation and a high incidence of non-random chromosomal abnormalities. Studying a patient with ataxia telangiectasia, Dr. Michael Davey, in collaboration with Dr. Ilan Krisch, has analyzed in molecular detail a T-cell chronic lymphocytic leukemia with a translocation between chromosome 14 q11, and the allelic chromosome 14 at band q32. He has shown that this represents a translocation that joins a J region gene of the T-cell receptor α locus with a putative cellular transforming gene at 14q32 that may contribute to the malignant transformation.

MOLECULAR ANALYSIS OF TRANSACTING FACTORS THAT MEDIATE LYMPHOID SPECIFIC GENE TRANSCRIPTION

As an approach to understanding development in the B lymphocyte lineage, Dr. Louis Staudt has characterized trans-acting factors that mediate lymphoid-specific gene transcription. He has studied the lymphoid-specific transcription of immunoglobulin (Ig) genes. The promoters of all Ig variable genes and Ig heavy chain enhancers contain a critical octamer DNA motif, ATTTGCAT. He demonstrated that this motif, together with a TATA motif, is necessary and sufficient for lymphoid-specific promoter activity. Dr. Staudt then assayed nuclear extracts of lymphoid and non-lymphoid cell lines for proteins which specifically bound to the octamer motif and detected two major species: a ubiquitous octamer binding protein (NF-A1) and a lymphoid-specific octamer binding protein (NF-A2). NF-A2 mediated the lymphoid-specific activity of Ig promoters. in order to test this hypothesis at a molecular level, he cloned a gene which is a strong candidate for the human NF-A2 structural gene. This was accomplished by screening a phage lambda gt11 expression library with a radiolabeled DNA probe containing the octamer motif. The cloned gene, termed

Oct-2, encodes an octamer-specific DNA binding protein which is itself lymphoid-specific in expression. This is the first gene to be cloned that encodes a tissue-specific DNA binding protein. By sequencing the oct-2 gene he identified a 60 amino acid domain that is homologous to the "homeo box" domain of proteins which are important for cell type determination and development in yeast and Drosophila. This domain of the oct-2 protein was shown to be required for DNA binding by site directed mutagenesis. This represents the first demonstration that a mammalian gene containing a homeo box domain is a sequence-specific DNA binding protein. The presence of the homeo box in the oct-2 gene product, is expressed at the earliest stages of lymphoid development and thus a study of the control of expression of the oct-2 gene will shed light on the process of commitment to the lymphoid lineage. Recent evidence indicates that the octamer motif may control the expression of other lymphoid-specific genes besides Ig, notably the BCL-2 gene involved in the pathogenesis of human follicular lymphomas. Using the cloned oct-2 gene, he should be able to determine the range of genes which are expressed exclusively in lymphocytes under the transcriptional control of the octamer motif.

THE GENETIC CONTROL OF THE IMMUNE RESPONSE

T lymphocytes, in contrast to antibodies, appear to recognize primarily a limited number of antigenic sites on any given antigenic protein. A primary goal of Dr. Jay Berzofsky's group has been to determine the rules that govern which structures on a protein molecule will be likely to comprise such immunodominant sites. Such rules would be valuable in understanding the processes of immune T-cell recognition as well as in identifying the appropriate components of artificial vaccines. In studying the frequency of T cells that respond to different antigenic sites, he found that a single site can so dominate the T-cell response that the presence or absence of a response to a single immunodominant site can make a difference between a high responder and a low responder even though low responders respond to other sites almost as well as high responders. The responses to these other sites never compensate. Therefore, there must be something unique about the immunodominant sites. Besides interaction with major histocompatibility (MHC) molecules, Dr. Berzofsky found that the mode by which the antigen is processed into fragments for T-cell recognition also determines which sites are seen. His data suggest that the products of natural processing of the protein are larger than the synthetic peptides and contain structures which hinder binding to certain MHC molecules or to the T-cell receptor. Besides MHC binding and antigen processing, a third factor in immunodominance is the intrinsic structure of the antigenic site. He showed previously that amphipathic helices have a higher than random chance of being immunodominant, and developed a computer program to locate such structures in protein amino acid sequences. He prospectively predicted sites in the malaria circumsporozoite protein and then tested overlapping peptides covering the whole molecule for their ability to elicit proliferation of T-cells from patients in an endemic area of West Africa. He and his coworkers found that the four most widely recognized sites were all predicted, and that only one strongly predicted site was not seen by this population. Similarly, he identified two helper T-cell sites from the HIV (AIDS virus) envelope, and has now shown that immunization with these peptides elicits enhanced antibody responses to the whole envelope when injected into monkeys. These sites were also recognized by human T cells from volunteers who had been immunized with a recombinant vaccinia virus expressing the HIV envelope. In mice, he identified other immunodominant sites in the envelope protein, including a very large one within which different MHC types of mice each recognize

different overlapping regions. Also, since cytotoxic T lymphocytes (CTL) may play a critical role in defense against AIDS, he used a recombinant vaccinia virus and transfectants expressing the HIV envelope gene to induce specific CTL against the HIV envelope. Using synthetic peptides, he was able to identify the first known CTL recognition site in the AIDS virus. However, such sites are very limited, and of five different class I MHC molecules that could have presented HIV envelope sites, only one appeared to be used. Hopefully these results may contribute to the design of a vaccine for AIDS.

SOMATIC GENE THERAPY FOR HUMAN GENETIC DISEASE

Dr. Michael Blaese has continued to focus on the development of techniques and reagents for use in the eventual application of gene therapy to human disease. A likely initial candidate disease in which gene therapy would be employed is the form of severe combined disease (SCID) caused by the deficiency of the purine salvage enzyme adenosine deaminase (ADA). Several systems have been studied including retroviral mediated gene transfer into bone marrow progenitor cells, cultured fibroblasts, and primary antigen specific IL-2 independent T cell lines. Dr. Blaese and his coworkers have been successful in transferring the gene for adenosine deaminase into ADA deficient T cell lines and totally correcting the biochemical defect in these cells in vitro. Using autologous bone marrow transplantation in lethally irradiated monkeys, he has also been successful in transferring the gene for human ADA into these monkeys. Expression of the human enzyme in the peripheral blood mononuclear cells of the recipient animals was detected within 45 days of transplantation and persisted for another 100 days. These results are very encouraging, but the levels of expression of human enzyme was low and not sustained for a long enough period to permit application of present techniques to the therapy of human disease. To attempt to avoid some of the problems encountered with bone marrow gene therapy, Dr. Blaese and his coworkers have been developing the approach of using cultured lymphocytes and fibroblasts as cellular vehicles for gene transfer. Cultured lymphocytes are readily infected with retroviral vectors and can be shown to be expressing the transferred gene appropriately before reintroduction into the body. The antigen specificity of lymphocytes can also be utilized to expand the population of gene transduced cells in vivo by simple immunization or to target the introduced gene to certain specific sites in the body such as tumor metastases. They have shown that murine antigen specific T cells transduced with the genes for neomycin resistance and human ADA will survive for weeks after reintroduction into intact animals and will continue to express both genes at high levels. Lymphocytes and fibroblasts transduced with the gene for growth hormone have been reintroduced into hypophectomized mice and rats and result in the production of growth hormone in these animals. Gene therapy combined with autologous bone marrow transplantation has many important advantages for the treatment of many human diseases, but it may well be that cultured lymphocytes and fibroblasts will see the first clinical application of gene transfer as a therapeutic procedure.

THE MULTI-CHAIN INTERLEUKIN-2 RECEPTOR ON NORMAL AND MALIGNANT LYMPHOCYTES

The success of the critical regulatory and effector functions of T lymphocytes requires that these cells change from a resting to an activated state. Antigen-stimulated activation of resting T cells induces the synthesis of interleukin-2 as well as high affinity IL-2 receptors that are not expressed on resting T cells. There are two classes of IL-2 receptors, one has a very high

affinity (10 pM dissociation constant (K_d) whereas the other has a much lower affinity (10 nM). Previously utilizing a monoclonal antibody, anti-Tac, as well as cross-linking studies with radiolabeled IL-2, Dr. Waldmann and his associates have identified two peptides that bind IL-2, the 55-kDa peptide reactive with the anti-Tac monoclonal, and a novel 70-75 kD(p75) peptide. Dr. Waldmann has proposed a multichain model for the high affinity IL-2 receptor in which both the p55 Tac and the p75 IL-2 binding peptides are associated in a receptor complex. Essentially all T cell functions require that the T cell be activated and expressed both the 55-kDa Tac peptide as well as the novel 75 kDa-IL-2 binding peptide. Large granular lymphocytes that are the precursors of lymphokine activated killer cells and natural killer cells present a different pattern. Dr. Waldmann and his coworkers have shown that such LAK and NK precursor cells express the p75 but not the 55-kDa IL-2 binding peptide. The interaction of IL-2 with the p75 peptide alone was shown to be sufficient to activate such cells as well as to induce such cells to transcribe mRNA encoding the Tac peptide.

Over the past year, Dr. Waldmann and his coworkers have developed evidence suggesting that a third 95-110 kDa peptide is associated with the Tac peptide in the high affinity receptor. Antibodies to this third peptide inconsistently co-precipitate the Tac peptide. Furthermore, flow-cytometric resonance energy transfer measurements support the association of the 95-110 kDa peptide with the Tac peptide on HUT 102 cells. Finally fluorescence photobleaching recovery measurements suggest that the Tac and the p95-110 peptides interact physically in situ on HUT-102 membranes.

Resting T cells do not express the Tac peptide. In contrast this peptide is expressed by a number of types of malignant mononuclear cells including human T cell lymphotrophic virus I, associated adult T cell leukemia cells, cutaneous T cell lymphoma cells, hairy cell B leukemia cells, as well as the Reed Sternberg cells of patients with Hodgkin's disease. In addition the Tac peptide is expressed by abnormally activated T cells involved in autoimmune disorders as well as by T cells participating in allograft rejection and in the graft vs., host reaction. Dr. Waldmann has exploited the fact that the Tac peptide of IL-2 receptors are present on abnormally activated T cells but not on normal resting cells to initiate clinical trials with IL-2 receptor directed therapy in patients with leukemia, or autoimmune diseases as well as those receiving allografts. Ten patients with HTLV-I induced adult T cell leukemia were treated with unmodified anti-Tac. None of the patients suffered untoward reactions. Furthermore, three of the patients had mixed, partial or complete remissions lasting from one to over ten months following anti-Tac therapy as assessed by routine hematological tests, immunofluorescence analysis of circulating cells, and molecular genetic analysis of HTLV-I provirus integration and of the T cell receptor gene rearrangement. In similar studies the administration of unmodified anti-Tac was associated with a prolongation of renal allograft survival in cynomolgus monkeys. In addition, in preliminary clinical trials, there was a virtual complete elimination of early renal allograft rejection episodes in patients who received anti-Tac in additional to conventional immunosuppressive therapy. In collaboration with Drs. FitzGerald and Pastan, clinical trials of Pseudomonas exotoxin conjugated anti-Tac have been initiated in patients with adult T-cell leukemia. In the initial studies these conjugates proved to be hepatotoxic. Subsequently a truncated 40-kDa form of Pseudomonas toxin (PE-40) has been generated that lacks the 18-kDa first domain that is involved in non-specific tissue binding. PE-40 conjugate of anti-Tac or interleukin-2 inhibited the protein synthesis of IL-2 receptor-expressing cells in vitro but were nontoxic to

normal lymphocytes. An effective form of IL-2 receptor-directed therapy involves radioisotopic chelates of anti-Tac produced in conjunction with Dr. Otto Ganzow. For example, the administration to subhuman primates of ^{90} yttrium anti-Tac led to a marked prolongation of the survival of cardiac xenografts when compared to the survival in untreated animals or in animals treated with unmodified anti-Tac. Finally, in collaboration with Dr. Cary Queen, Dr. Waldmann and his coworkers have generated recombinant antibodies that have the variable region of the mouse anti-Tac monoclonal antibody joined at a molecular level to the human constant kappa light chain and IgG1 or IgG3 heavy chain regions. These "humanized" antibodies retained their specificity and affinity of binding to Tac expressing cells and were as effective as native anti-Tac in inhibiting antigen and mixed leukocyte-induced T cell proliferation. Thus the development of monoclonal antibodies directed toward the IL-2 receptor expressed on adult T cell leukemia T cells, on autoreactive T cells of certain patients with autoimmune disorders, and on host T cells responding to foreign histocompatibility antigens on organ allografts may provide a rational therapeutic approach for these clinical conditions.

Dr. David Nelson has demonstrated a soluble form of the P55 component (Tac protein) of the human interleukin-2 receptor in the supernatants of activated T-cells, B-cells, and monocytes *in vitro* and in the serum of normal individuals *in vivo*. He introduced an enzyme-linked immunosorbent assay procedure for the measurement of the Tac peptide in the serum. Elevated levels of soluble Tac protein were found in the sera of patients with human diseases including the adult T-cell leukemia (ATL), Tropical Spastic paraparesis, and the acquired immunodeficiency syndrome (AIDS) as well as in hairy cell leukemia. In patients with adult T cell leukemia and hairy cell leukemia, the serum levels of Tac protein were indicative of tumor burden and a favorable response to therapy was associated with reductions in the serum level of Tac protein. Elevations of Tac protein in serum were also associated with allograft rejection episodes in patients with liver and heart-lung transplants. Patients with certain autoimmune diseases also had elevated levels of Tac protein in serum and joint fluids. In summary, the measurement of soluble Tac protein in various body fluids has been shown by Dr. Nelson and his coworkers to be useful in the diagnosis and management of certain cancer patients as well as in monitoring immune mediated events *in vivo*.

ISOLATION AND CHARACTERIZATION OF BIOLOGICAL RESPONSE MODIFIERS AND OLIGOSACHARIDES THAT REGULATE THE HUMAN IMMUNE RESPONSE

Dr. Andrew Muchmore has been pursuing the purification, immunologic characterization, sequencing and cloning of uromodulin, an 85 KD immunosuppressive glycoprotein derived from pregnancy urine. Uromodulin is a heavily glycosylated single chain protein composed of approximately 30% carbohydrate. He is now focusing on the characterization of glycosylation patterns responsible for the biologic activity of uromodulin. Utilizing a combination of protease digestion and multiple chromatographic steps, Dr. Muchmore has purified to homogeneity and structurally characterized by ^{1}H NMR two biologically active high mannose oligosacharides. Based on these structural observations, alternative sources for active oligosaccharides have been found and include egg white protein, soybean meal, and yeast mannan. These compounds inhibit or enhance the immune response both *in vitro* and *in vivo*. Utilizing defined oligosaccharides ranging from 7-11 sugar residues, Dr. Muchmore now performed a number of structure-function studies. The compounds studied were

shown to inhibit T cell responsiveness, enhance PGE_2 synthesis, induce collagenase release, block hydrogen peroxide formation, activate the hexose monophosphate shunt, bind to IL-1, TNF, and IL-2, and regulate the expression of class II MHC molecules. Dr. Muchmore has applied for a patent covering the immunoregulatory activity of these structurally unique compounds.

INSULIN-LIKE GROWTH FACTOR (IGF-I AND IGF-II) RECEPTORS

The insulin-like growth factors (IGF-I and IGF-II) play an important role in the normal growth process in vivo. Molecular cloning of the human type II IGF receptor by Rutter and his collaborators revealed an 80% sequence homology with the bovine cation independent mannose 6-phosphate (man-6-P) receptor suggesting that this receptor is multifunctional, binding both IGF-II and lysosomal enzymes. Dr. Peter Nissley and his associates have performed biochemical experiments which support this proposal. Rat liver type II IGF receptor, purified by the conventional method of IGF-II affinity chromatography, bound quantitatively to a ß-galactosidase affinity column and was eluted with Man-6-P. Bovine liver Man-6-P receptor prepared using the conventional method of affinity chromatography on phosphomannan-Sepharose, bound IGF-II with high affinity (Kd=1nM). For immunologic studies, Dr. Nissley purified type II IGF receptors and Man-6-P receptors in parallel from rat placental membranes using either IGF-II- or ß-galactosidase affinity chromatography. A panel of 5 antisera that previously had been raised against either type II IGF receptor or Man-6-P receptor behaved identically toward type II receptor versus Man-6-P receptor in ligand blocking and immunoprecipitation assays. Dr. Nissley performed additional experiments to define interactions of the two classes of ligands (IGFs and lysosomal enzymes) for binding to the IGF-II/Man-6-P receptor. He found that a panel of lysosomal enzymes inhibited the binding of ^{125}I-IGF-II to the purified receptor and that this inhibition resulted from a change in the binding affinity for IGF-II. He also examined the ability of IGF-II to modulate the binding of radiolabeled lysosomal enzyme (ß-galactosidase) to the receptor. IGF-II inhibited the binding of ^{125}I-ß-galactosidase to purified receptor and also inhibited the uptake of ^{125}I-ß-galactosidase in two cell lines in culture (C6 and BRL-3A) via the IGF-II/Man-6-P receptor. Additionally, he has examined the developmental pattern of IGF-II/Man-6-P receptor expression by extracting rat tissues and performing western blotting using an antisera which is highly specific for the IGF-II/Man-6-P receptor. He found that the receptor is present in multiple tissues at levels as high as 1% of extractable protein and that the receptor is developmentally regulated, being high in fetal tissues and declining postnatally to much lower levels by day 20.

REGULATORY FUNCTION OF PROLINE AND ITS METABOLITE PYRROLINE-5-CARBOXYLATE

Dr. James Phang previously proposed that pyrroline-5-carboxylate (P5C) is an intercellular communicator. Dr. Phang has now established that P5C circulates as a constituent of human plasma and importantly, the levels rise to nutrient-dependent peaks. Temporally, these peaks occurred 2-3 hours following meals. In the face of these marked changes in plasma P5C levels, little variation was seen in plasma amino acids. With fasting, the peaks were markedly attenuated. Since only polypeptide hormones undergo such fluctuations, the observed changes in plasma P5C are consistent with a communicator role for this molecule.

Dr. Phang identified a number of specific inhibitors of P5C uptake. P5C enters cells by a group translocation mechanism coupled to the transfer of

oxidizing potential and thereby initiates a cascade stimulating PRPP and ribonucleotide synthesis. This novel uptake mechanism may be central to the previously demonstrated regulatory effects of P5C on cultured cells. Dr. Phang identified three specific inhibitors of P5C uptake. They are pyridine-2-carboxaldehyde, pyridine-2, 6-dicarboxaldehyde and picolinic acid which inhibited P5C uptake with Ki's of 0.6 mM, 0.3 mM, and 5.0 mM, respectively. All three inhibitors were competitive with P5C and the inhibition was specific in that other amino acid uptake systems (A, L, ASC) were unaffected.

P5C reductase may be a component of the aforementioned group translocation mechanism. Therefore, Dr. Phang purified P5C reductase from human red cells. Since the group translocation mechanism transfers electrons from the NADPH system, he directly assessed the relative oxidation of NADH and NADPH by this enzyme. Dr. Phang devised a double label method using NADPH tritiated in the exchangeable position and uniformly labeled ^{14}C-P5C. With P5C, NADH, and NADPH at physiologic concentrations, only NADPH was oxidized by P5C reductase. More importantly, at saturating concentrations of NADH and P5C, NADPH was preferentially oxidized at the expense of proline production. This occurred because proline production by the NADPH-mediated reaction has a lower Vmax. From these studies he concluded that P5C reductase can be considered an NADPH-oxidase which uses P5C as an electron acceptor. The traditional role of proline synthesis is reserved for the proline-inhibitable, NADH-utilizing enzyme in certain tissues.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04002-19 MET

PERIOD COVERED: October 1, 1987 through September 30, 1988

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 Dysfunctions

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

PI:	Thomas A. Waldmann, M.D.	Branch Chief	MET, NCI
	Michael Davey, M.D.	Medical Staff Fellow	MET, NCI
	Steven J. Greenberg, M.D.	Medical Staff Fellow	MET, NCI
	Jack Burton, M.D.	Guest Researcher	MET, NCI
	Richard P. Junghans, M.D.	Medical Staff Fellow	MB, DCT, NCI
	Colin Glenn Begley, M.D.	Visiting Fellow	MET, NCI

COOPERATING UNITS (if any)
Laboratory of Molecular Biology, NCI
Radiation Oncology Branch, NCI

LAB/BRANCH
Metabolism Branch

SECTION

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8	6	2

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors "B" 100%
☐ (a2) Interviews

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

Resting T cells do not express high-affinity IL-2 receptors, but receptors are rapidly expressed on T cells after activation with antigen or mitogen. There are two classes of IL-2 receptors that differ in their affinity for IL-2, including one with a very high affinity and the other with a much lower affinity. In a major development, a novel 75-kDa non-Tac IL-2 binding peptide was identified. Cell lines bearing either the 55-kDa Tac or the p75 peptide alone manifested low-affinity IL-2 binding, whereas cell lines bearing both peptides manifested both high- and low-affinity receptors. Fusion of cell membranes from low-affinity IL-2 binding cells bearing the Tac peptide alone with membranes from a cell line bearing the p75 peptide alone generated hybrid membranes bearing high-affinity receptors. These observations suggested a multichain model for the high-affinity IL-2 receptor in which both the p55 Tac and the p75 IL-2 binding peptides are associated in a receptor complex. Essentially all T-cell functions require that the T cell be activated and express both the 55-kDa Tac peptide as well as the novel 75-kDa IL-2 binding peptide. The pattern with lymphokine-activated killer (LAK) cells and natural killer (NK) cells is quite different. Over the past year, it was shown that such LAK and NK precursor cells, as well as different types of leukemias of large granular lymphocytes with NK activity, express the p75 but not the 55-kDa IL-2 binding peptide. Evidence has been presented suggesting that a third 95-110 kd peptide is an associated participant in the multichain IL-2 receptor. In contrast to resting cells, certain leukemic T cells, T cells involved in autoimmune disorders and in allograft rejection express the Tac peptide. To exploit this differential expression, clinical trials of patients with Tac positive adult T-cell leukemia have been initiated using unmodified anti-Tac toxin conjugated and isotope chelated anti-Tac.

GPO 914-918

Project Description

Major Findings:

The success of the critical regulatory and effector functions of T lymphocytes requires that these cells change from a resting to an activated state. Antigen-stimulated activation of resting T cells induces the synthesis of interleukin-2 as well as high affinity IL-2 receptors that are not expressed on resting T cells. There are two classes of IL-2 receptors, one has a very high affinity (10 pM dissociation constant (K_d) whereas the other has a much lower affinity (10 nM). Previously utilizing a monoclonal antibody, anti-Tac, as well as cross-linking studies with radiolabeled IL-2, Dr. Waldmann and his associates have identified two peptides that bind IL-2, the 55-kDa peptide reactive with the anti-Tac monoclonal, and a novel 70-75 kD(p75) peptide. Dr. Waldmann has proposed a multichain model for the high affinity IL-2 receptor in which both the p55 Tac and the p75 IL-2 binding peptides are associated in a receptor complex. Essentially all T cell functions require that the T cell be activated and expressed both the 55-kDa Tac peptide as well as the novel 75 kDa-IL-2 binding peptide. Large granular lymphocytes that are the precursors of lymphokine activated killer cells and natural killer cells present a different pattern. Dr. Waldmann and his coworkers have shown that such LAK and NK precursor cells express the p75 but not the 55-kDa IL-2 binding peptide. The interaction of IL-2 with the p75 peptide alone was shown to be sufficient to activate such cells as well as to induce such cells to transcribe mRNA encoding the Tac peptide.

Over the past year, Dr. Waldmann and his coworkers have developed evidence suggesting that a third 95-110 kDa peptide is associated with the Tac peptide in the high affinity receptor. Antibodies to this third peptide inconsistently co-precipitate the Tac peptide. Furthermore, flow-cytometric resonance energy transfer measurements support the association of the 95-110 kDa peptide with the Tac peptide on HUT 102 cells. Finally fluorescence photobleaching recovery measurements suggest that the Tac and the p95-110 peptides interact physically in situ on HUT-102 membranes.

Resting T cells do not express the Tac peptide. In contrast this peptide is expressed by a number of types of malignant mononuclear cells including human T cell lymphotrophic virus I, associated adult T cell leukemia cells, cutaneous T cell lymphoma cells, hairy cell B leukemia cells, as well as the Reed Sternberg cells of patients with Hodgkin's disease. In addition the Tac peptide is expressed by abnormally activated T cells involved in autoimmune disorders as well as by T cells participating in allograft rejection and in the graft vs., host reaction. Dr. Waldmann has exploited the fact that the Tac peptide of IL-2 receptors are present on abnormally activated T cells but not on normal resting cells to initiate clinical trials with IL-2 receptor directed therapy in patients with leukemia, or autoimmune diseases as well as those receiving allografts. Ten patients with HTLV-I induced adult T cell leukemia were treated with unmodified anti-Tac. None of the patients suffered untoward reactions. Furthermore, three of the patients had mixed, partial or complete remissions lasting from one to over ten months following anti-Tac therapy as assessed by routine hematological tests, immunofluorescence analysis of circulating cells, and molecular genetic analysis of HTLV-I provirus integration and of the T cell

receptor gene rearrangement. In similar studies the administration of unmodified anti-Tac was associated with a prolongation of renal allograft survival in cynomolgus monkeys. In addition, in preliminary clinical trials, there was a virtual complete elimination of early renal allograft rejection episodes in patients who received anti-Tac in additional to conventional immunosuppressive therapy. In collaboration with Drs. FitzGerald and Pastan, clinical trials of Pseudomonas exotoxin conjugated anti-Tac have been initiated in patients with adult T-cell leukemia. In the initial studies these conjugates proved to be hepatotoxic. Subsequently a truncated 40-kDa form of Pseudomonas toxin (PE-40) has been generated that lacks the 18-kDa first domain that is involved in non-specific tissue binding. PE-40 conjugate of anti-Tac or interleukin-2 inhibited the protein synthesis of IL-2 receptor-expressing cells in vitro but were nontoxic to normal lymphocytes. An effective form of IL-2 receptor-directed therapy involves radioisotopic chelates of anti-Tac produced in conjunction with Dr. Otto Ganzow. For example, the administration to subhuman primates of ^{90} yttrium anti-Tac led to a marked prolongation of the survival of cardiac xenografts when compared to the survival in untreated animals or in animals treated with unmodified anti-Tac. Finally, in collaboration with Dr. Cary Queen, Dr. Waldmann and his coworkers have generated recombinant antibodies that have the variable region of the mouse anti-Tac monoclonal antibody joined at a molecular level to the human constant kappa light chain and IgG1 or IgG3 heavy chain regions. These "humanized" antibodies retained their specificity and affinity of binding to Tac expressing cells and were as effective as native anti-Tac in inhibiting antigen and mixed leukocyte-induced T cell proliferation. Thus the development of monoclonal antibodies directed toward the IL-2 receptor expressed on adult T cell leukemia T cells, on autoreactive T cells of certain patients with autoimmune disorders, and on host T cells responding to foreign histocompatibility antigens on organ allografts may provide a rational therapeutic approach for these clinical conditions.

Honors and Awards:

1987 CIBA-GEIGY Drew Award in Biomedical Research
1987 Morris F. Shaffer Lectureship, Tulane Medical School
1987 Simon Shubitz Prize for Cancer Research, Univ. of Chicago Medical School
1988 Dorothy Snider Foundation Lecture on Cancer Research

Publications:

Waldmann TA. IL-2 receptor expression: Role in the proliferation of normal and malignant lymphocytes. In: Johnson I, Rue Y, eds. 1987 Royal Society of Medicine Services International Congress and Symposium Series 121, A Perspective on Biology and Medicine in the Twenty-First Century 1987;113-122.

Waldmann TA. The interleukin-2 receptor on normal and malignant lymphocytes. In: Gupta S, Fauci AS, Paul W, eds. Mechanisms of Lymphocyte Activation and Immune Regulation. NY Elsevier Science Pub Co 1987;129-138.

Waldmann TA. The arrangement of immunoglobulin and T-cell receptor genes in human lymphoproliferative diseases. Adv Immunol 1987;40:247-321.

Szöllösi J, Damjanovich S, Goldman CK, Fulwyler MJ, Aszalos AA, Goldstein G, Rao P, Talle MA, Waldmann TA. Flow cytometric resonance energy transfer measurements support the association of a 95-kDa peptide termed T27 with the 55-kDa Tac peptide. Proc Natl Acad Sci USA 1987;84:7246-51.

Tsudo M, Goldman CK, Bongiovanni KF, Chan WC, Winton EF, Yagita M, Grim EA, Waldmann TA. The p75 peptide is the receptor for interleukin-2 expressed on large granular lymphocytes and is responsible for the interleukin-2 activation of these cells. Proc Natl Acad Sci USA 1987;84:5394-98.

Waldmann TA, Tsudo M. T-cell proliferation and the multichain IL-2 receptor complex. In: Gale RP, Golde DW, eds. Recent Advances in Leukemia and Lymphoma. NY Alan R Liss 1987;329-38.

Kozak RW, Haskell JF, Greenstein LA, Rechler MM, Waldmann TA, Nissley SP. Type I and II insulin-like growth factor receptors on human activated lymphocytes. Cell Immunol 1987;109:318-31.

Waldmann TA. The role of multichain IL-2 receptor complex in the control of normal and malignant T-cell proliferation. In: Environmental Health Perspectives (EHP) 1987;75:11-15.

Waldmann TA. The multichain interleukin-2 receptor: from the gene to the bedside. In: Harvey Lecture Series 1988;82:1-17.

Waldmann TA, Tsudo M. The role of the multichain interleukin-2 receptor in the control of normal and malignant T cell proliferation. In: Hankins D, Puch D, eds. Hormones Cell Biology and Cancer. NY Alan R Liss, Perspectives and Potentials 1988;262:283-93.

Waldmann TA, Goldman CK, Tsudo M. The molecular biology of the multichain interleukin-2 receptor. In: Mani JC, Dornand J, eds. Lymphocyte Activation and Differentiation. NY Walter de Greiyter and Co. 1988:133-142.

Wright JJ, Birx DL, Wagner DK, Waldmann TA, Blaese RM, Fleisher TA. HIV infection and normalization of antibody responsiveness in a patient with common variable hypogammaglobulinemia. N Engl J Med 1987;317:1516-20.

Kaulfersch W, Baker JR Jr, Burman KD, Ahmann AJ, Davis JC, Waldmann TA. Immunoglobulin and T cell antigen receptor gene arrangements indicate that the immune response in autoimmune disease is polyclonal in nature. J Clin Endocrinol Metab 1988;66:958-63.

Waldmann TA, Kozak RW, Tsudo M, Oh-ishi T, Bongiovanni KF, Goldman CK. Receptors in adult T-cell leukemia: a target for immunotherapy. In: Neth R, Gallo RC, Greaves MF, Kabisch H, eds. Hematology and Blood Transfusion Vol XXXI, Heidelberg, Springer-Verlag, 1987:110-115.

Waldmann TA. Intestinal lymphanglectasia. In: Birth Defects Compendium, 3rd Edition 1988, in press.

Waldmann TA. The primary and acquired immunodeficiency diseases. In: Sampter M, ed. The Immunological Diseases. Boston, Little Brown & Co 1988, in press.

Reed MH, Shapiro ME, Strom TB, Milford EL, Carpenter CB, Litrin NL, Waldmann TA, Kirkman RL. Prolongation of primate renal allografts with anti-Tac monoclonal antibody. Curr Surj 1988, in press.

Waldmann TA, Goldman CK, Tsudo M. The multichain IL-2 receptor: from the gene to the bedside. In: Sourerijn JH, ed. XIII International Congress of Clinical Chemistry 1988, in press.

Oh-ishi T, Goldman CK, Misiti J, Waldmann TA. The role of interleukin-2 in the differentiation of suppressor T-cells: The effect of blockade of the interleukin-2 receptor by anti-Tac antibody. Proc Natl Acad Sci 1988, in press.

Chan WC, Dahb C, Waldmann T, Link S, Maule A, Nicholson J, Bach F, Bongiovanni K, McLue P, Winton EF. Large granular lymphocyte proliferation: An analysis of T-cell receptor gene arrangement and expression and the effect of in vitro culture with inducing agents. J Immunol 1988, in press.

Kaulfersch W, Fiocchi C, Waldmann TA. Immunoglobulin and T cell antigen receptor gene rearrangements indicate that lamina propria lymphocyte populations in inflammatory bowel disease are polyclonal. Gastroenterology 1988, in press.

Edidin M, Aszalos A, Damjanovich S, Waldmann TA. Lateral diffusion of the Tac antigen of the IL-2 receptor complex and T27, a 95-kD peptide associated with Tac in the membrane of the HUT 102-B2 T cell line. J Immunol 1988, in press.

Waldmann TA. Disorders of IL-2 receptor expression in HTLV-I-associated adult T-cell leukemia. In: Pullman ME, ed. Retroviruses and Disease. San Diego CA, Academic Press 1988, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04004-26

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Amino Acids and Growth Factors in Cell Activation

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

PI:	James M. Phang	Section Head	MET, NCI
	A. James Mixson	Medical Staff Fellow	MET, NCI
	Patricia Cortazar	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

DCBD, NCI, NIH, Bethesda, Maryland

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" 100%

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

We have established that pyrroline-5-carboxylate (P5C) is a nutrition-dependent intercellular communicator which modulates the effects of certain growth factors in transmembrane signaling. First, we demonstrated that P5C is a constituent of human plasma and levels of P5C show peaks 15-fold over baseline approximately 2 hrs. following meals. Thus, the physiologic variation in P5C concentrations can transmit nutrient-dependent information to peripheral tissues. The interaction of P5C with cells occurs through a novel mechanism. P5C entry into cells is mediated by a specific, saturable group translocation mechanism which transfers oxidizing potential across cell membranes. This transfer of oxidizing potential initiates a metabolic cascade which sequentially activates the pentose phosphate shunt, stimulates phosphoribosyl pyrophosphate formation and increases ribonucleotide synthesis. P5C reductase plays a role in the group translocation and transfer of oxidizing potential. The enzyme has been purified from human erythrocytes and its kinetic properties defined. From these properties it can be concluded that the enzyme functions as an NADPH oxidase with P5C as electron acceptor. Furthermore, this enzyme is associated with plasma membranes and the transfer of oxidizing potential is regulated by protein-protein interactions. Of special interest, P5C acts synergistically with platelet-derived growth factor in stimulating phosphoribosyl pyrophosphate formation. The mechanism involves the turnover of phosphoinositides and activation of protein kinase C. These studies show that P5C serves as a novel mechanism for integrating nutritional influences with growth factor-stimulated cellular events to produce physiologic or pathophysiologic responses.

GPO 914-918

Project Description

Major Findings:

1. Nutrient-dependent Peaks of P5C in Human Plasma

We previously proposed that pyrroline-5-carboxylate (P5C) is an intercellular communicator. We have now established that P5C circulates as a constituent in human plasma and importantly, the levels rise to nutrient-dependent peaks. Using a sensitive, specific radioenzyme method for P5C, we defined normal levels in 46 normal volunteers. P5C levels were 0.39 $^{+}$ .09 uM (mean $^{+}$ std. dev.). Importantly we have identified marked diurnal variation in plasma P5C levels. In 5 normal volunteers on ad libitum hospital diets, venous P5C levels peaked as much as 15-fold over baseline levels. The nadir occurred between midnight and 6 a.m. and several peaks were seen during the day with the highest peak occurring in the late P.M. Temporally, these peaks occurred 2-3 hours following meals. In the face of these marked changes in plasma P5C levels, little variation was seen in plasma amino acids. With fasting, the peaks in P5C were markedly attenuated. Since polypeptide hormones are the only plasma constituents known to exhibit such fluctuations, the observed changes in plasma P5C are consistent with a communicator role for this molecule.

2. Recent Studies on P5C Uptake

a. Inhibitors

We have identified a number of specific inhibitors of P5C uptake. P5C enters cells by a group translocation mechanism coupled to the transfer of oxidizing potential and thereby initiates a cascade for stimulating PRPP and ribonucleotide synthesis. This novel uptake mechanism may be central to the previously demonstrated regulatory effects of P5C on cultured cells. We have identified three specific inhibitors of P5C uptake. They are pyridine-2-carboxaldehyde, pyridine-2, 6-dicarboxaldehyde and picolinic acid which inhibited P5C uptake with Ki's of 0.6 mM, 0.3 mM and 5.0 mM, respectively. All three inhibitors were competitive with P5C and the inhibition was specific in that other amino acid uptake systems (A, L, ASC) were unaffected. We have also identified a nonspecific inhibitor, p-chloromercuribenzosulfonate (PCMBS) which acts by binding to thiol groups on cell surface proteins. Interestingly, P5C uptake is extremely sensitive to this inhibitor. PCMBS abolished P5C uptake at concentrations (15 uM) 2 logs lower than that necessary for blocking the uptake of N-methyl aminoisobutyric acid, the model amino acid for system A. Furthermore, the presence of P5C or one of the specific inhibitors protected against the effects of PCMBS. Assuming that PCMBS forms an adduct with thiols on the putative P5C permease, these findings may allow for identification and purification of this membrane protein by using radioactively labeled PCMBS as a marker.

b. P5C Uptake and Multiple Drug Resistance

Previous studies identified differences between parent wild type MCF-7 cells and adriamycin resistant cells (AdrR) in their P5C-dependent activation of redox mechanisms. We have shown that this difference is due to marked

differences in P5C uptake between MCF-7 and AdrR. In kinetics studies, we showed that the saturable P5C uptake mechanism is virtually absent in MCF-7 whereas it is at levels in AdrR comparable to that found in other cultured cells. In spite of this marked difference in P5C uptake, other transport systems for amino acids (A, ASC, L) are similar in the two cell lines. The characteristics of P5C uptake in AdrR, e.g. Km for P5C, sodium independence, and the lack of inhibition by other amino acids, are identical to those defined in Chinese hamster ovary cells. Our current studies are focusing on the mechanism relating P5C uptake to the mechanism(s) of multiple drug resistance.

3. Properties of P5C Reductase - Association with Subcellular Organelles

P5C reductase may be a component of the aforementioned group translocation mechanism. Therefore, we purified P5C reductase from human red cells. Since the group translocation mechanism transfers electrons from the NADPH system, we directly assessed the relative oxidation of NADH and NADPH by this enzyme. We devised a double label method using NADPH tritiated in the exchangeable position and uniformly labeled 14C-P5C. With P5C, NADH, and NADPH at physiologic concentrations, only NADPH was oxidized by P5C reductase. More importantly, at saturating concentrations of NADH and P5C, NADPH was preferentially oxidized at the expense of proline production. This occurred because proline production by the NADPH-mediated reaction has a lower Vmax. From these studies we condlude that P5C reductase can be considered an NADPH-oxidase which uses P5C as electron acceptor rather than a proline synthetic enzyme which preferentially uses NADPH. Importantly although P5C reductase is a cytosolic enzyme, it is associated with subcellular components. Using sucrose density gradients, we found that P5C reductase activity is associated with mitochondria and plasma membranes. The activity associated with plasma membranes has a markedly higher affinity for NADPH than for NADH. This association with plasma membranes and the preferential transfer of oxidizing potential to the NADP-NADPH redox system supports a role for P5C reductase in the group translocation mechanism and explains the previously demonstrated effects of added P5C in regulating PRPP and ribonucleotide synthesis.

Publications:

Mixson AJ, Phang JM. The uptake of pyrroline-5-carboxylate. Group translocation mediating the transfer of oxidizing potential. J Biol Chem 1988, in press.

Yeh GC, Phang JM. Stimulation of phosphoribosyl pyrophosphate and purine nucleotide production by pyrroline-5-carboxylate in human erythrocytes. J Biol Chem 1988, in press.

Phang JM, Scriver CR. Disorders of proline and hydroxyproline metabolism. In: The Metabolic Basis of Inherited Disease. Scriver CR, Beaudet AL, Sly WS, Valle D, Eds. 6th ed. New York: McGraw-Hill 1988, in press.

Flynn MP, Martin MC, Moore PT, Stafford JA, Fleming GA, Phang JM. Type II hyperprolinaemia in a pedigree of Irish travellers (nomads). Arch Dis Childhood London 1988, in press.

Fleming GA, Granger A, Rogers DR, Prosser M, Ford DB, Phang JM. Diurnal peak plasma pyrroline-5-carboxylate concentrations exceed basal levels by multiple folds. J Clin Endo Met 1988, submitted.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04015-17 MET

PERIOD COVERED
October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development and Function of Humoral and Cellular Immune Mechanism

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

R. Michael Blaese	Section Chief	MET, NCI
Ken Culver	Medical Staff Fellow	MET, NCI
Thomas Fleisher	Senior Investigator	CPD, CC
Robert Moen	Senior Staff Fellow	LMN, NHLBI
French Anderson	Chief	LMH, NHLBI
Alan Palestine	Senior Investigator	NEI
Michael Wood	Howard Hughes Scholar	MET, NCI
Ahron Friedberg	Howard Hughes Scholar	MET, NCI

COOPERATING UNITS *(if any)*
LMH, NHLBI

LAB/BRANCH
Metabolism Branch

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	2	3

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors "B" 100%
☐ (a2) Interviews

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

The development of techniques to facilitate gene therapy in man has continued to be a major emphasis of our work. Using a retroviral gene transfer system we have successfully transferred the gene for adenosine deaminase into ADA deficient T cells in vitro and totally corrected the biochemical defect in these cells. Using a bone marrow transplantation model in monkeys, we have reconstituted irradiated monkeys with autologous marrow which has been treated with the human ADA retrovirus vector. The reconstituted monkeys were found to express human ADA in their peripheral blood cells for up to 120 days after reconstitution. The level and duration of expression are insufficient to attempt human reconstitution at this time but the results suggest that this approach may ultimately provide a successful mechanism for the treatment of a variety of genetic disorders of man. We have also used cultured lymphocytes and fibroblasts as cellular vehicles for gene transfer in animals. With T cells as the carriers of transduced genes, mice have continued to express human ADA, rat growth hormone, and neomycin phosphotransferase for many weeks and therefore studies with lymphocytes as gene therapy targets will continue to be a major project. We have developed a test to detect the carriers of the Wiskott-Aldrich syndrome, an X-linked immunodeficiency disorder, based on the finding that these females have a markedly unbalanced pattern of X-chromosome inactivation in their T cells, B cells, and PMNs. Our studies developing and defining the new immunosuppressive drug succinylacetone (SA) have continued. We have found that this agent is perhaps the most potent T and B cell immunosuppressive compound available, totally blocking allograft rejection and graft-versus-host disease (GVHD) in F1 animals and preventing GVHD during total allogeneic bone marrow transplantation while allowing normal engraftment and reconstitution to occur. SA is also a very potent inhibitor of both primary and secondary antibody responses. Trials of SA in a rat model of autoimmune eye disease have shown that it is remarkably effective in preventing a type of uveitis that usually results in blindness in experimental animals.

Project Description

Major Findings:

A major effort of the Cellular Immunology Section has continued to focus on the development of techniques of gene transfer which will be useful for the eventual application of gene therapy in man. Several systems have been studied including retroviral mediated gene transfer into bone marrow progenitor cells, cultured fibroblasts, transformed T cells and primary antigen specific IL-2 independent T cell lines from mice, rats, monkeys and IL-2 dependent T cells from man. We have been successful in transferring the gene for adenosine deaminase into ADA deficient T cell lines and totally correcting the biochemical defect in these cells in vitro. Using autologous bone marrow transplantation in lethally irradiated monkeys, we have also been successful in transferring the gene for human ADA into these monkeys. Expression of the human enzyme in the peripheral blood mononuclear cells of the recipient animals was detected within 45 days of transplantation and persisted for another 100 days. These results are very encouraging, but the level of expression of human enzyme was low and not sustained for a long enough period to permit application of present techniques to the therapy of human disease. To attempt to avoid some of the problems encountered with bone marrow gene therapy we have been developing the alternative approach of using lymphocytes and fibroblasts as cellular vehicles for gene transfer. Lymphocytes have several potential advantages for gene therapy. Cultured lymphocytes are readily infected with retroviral vectors, can be selected for appropriate gene expression in culture, and can be thoroughly tested before reintroduction into the body. The antigen specificity of lymphocytes can also be utilized to expand the population of gene transduced cells in vivo by simple immunization or to target the introduced gene to certain specific sites in the body such as directing antineoplastic factors to the sites of tumor metastases. We have shown that murine antigen specific T cells transduced with the genes for neomycin resistance and human ADA will survive for many weeks after reintroduction into intact animals and will continue to express both genes at high levels. Lymphocytes and fibroblasts transduced with the gene for growth hormone have been reintroduced into hypophectomized mice and rats and result in the production of growth hormone in these animals. Gene therapy combined with autologous bone marrow transplantation will be important for the treatment of many inherited human diseases, but it may well be that cultured lymphocytes and/or fibroblasts will be the first cellular vehicles bringing clinical application of gene transfer as a therapeutic procedure.

We have continued with our long time interest in the Wiskott-Aldrich syndrome with studies of platelet function before and after splenectomy, changes in platelet cell membrane glycoproteins occurring after splenectomy, detailed lymphocyte phenotype analysis of both T cells and B cells, family genetic linkage analysis using X-chromosome RFLPs, and carrier detection based on the pattern of X-inactivation found in peripheral cells of the carriers. This latter study is based on the observation that the affected boys have defects in T cell, B cell, monocyte, granulocyte, and platelet function and yet the carrier females have no detectable defects in any of these lineages. Since all females are mosaics for traits inherited on the X-chromosome because of random inactivation of one of her two X-chromosomes (Lyonization) occurring in the early embryo, carriers might be expected to demonstrate abnormalities unless all

their cells were derived from precursors in which the chromosome carrying the defective gene was inactivated. We have studied this question using RFLPs for the probes to the X-linked genes PGK and HPRT to distinguish the maternal from the paternally inherited X-chromosome and methylation sensitive restriction endonucleases to distinguish the active from the inactive X-chromosomes. Whereas approximately half the cells from normal females express the genes on the paternally derived X and the remaining express genes from the maternally derived X-chromosome, the T cells, B cells, and granulocytes from obligate carriers of the WAS gene were found to be using only one of the two chromosomes in all their peripheral cells. This strikingly unbalanced pattern of X-inactivation was not seen in any of the more than 80 normal females studied and thus appears to represent a reliable marker for the carrier state in these females.

Our studies of the immunosuppressive compound succinylacetone have continued to provide exiting information about this most potent of immunosuppressive agents. Succinylacetone is a seven carbon organic acid. It is an inhibitor of the second step of heme biosynthesis and has been shown to have inhibitory effects on the growth of certain tumors in vitro. We initially demonstrated that the compound appeared to have potent immunosuppressive activity in the rat because it was capable of preventing the rejection of tumor allografts. More recent studies showed that succinylacetone behaves as a profound suppressive agent for T-cell-mediated immune functions. It not only blocks tumor and other forms of allograft rejection, but it also prevents graft-versus-host disease induced in adult F1 animals after the injection of parental strain lymphoid cells. In a model of bone marrow transplantation in fully allogeneic adult rats, succinylacetone treatment not only prevented lethal graft-versus-host disease in the transplanted animals but also permitted normal engraftment and full reconstitution. The striking finding is that the treatment required to prevent graft-versus-host disease was limited to 24 days of duration and yet the animals remained fully chimeric for at least 300 days post bone marrow transplantation. Immune competence returned to the transplanted animals in a timely fashion, and survival in the recipients of allogeneic cells was equivalent to the survival in transplant recipients receiving syngeneic bone marrow. Succinylacetone has been shown to have profound effects on the B-lymphocyte system as well. Initially, it was shown to inhibit the production of antibodies to sheep erythrocytes in rats treated for a period of two weeks. We have extended these initial studies to demonstrate that the drug has immunosuppressive effects in mice as well. Furthermore we have shown, using a series of model antigens that are both helper T-cell-dependent and helper T-cell-independent type I and type II antigens, that succinylacetone interferes with the production of antibodies to all classes of antigen. Therefore its inhibitory effects cannot be explained solely by the inhibition of T-cell function. Thus succinylacetone is one of the very few agents to have profound effects on the production of antibodies and immunoglobulin. In a series of collaborative studies with Dr. Alan Palestine of the National Eye Institute, we have been treating Lewis rats that have been injected with purified retinal protein that induces a model disorder of inflammatory uveitis. One hundred percent of Lewis rats develop blindness within 14 days of treatment with Freund's containing purified retinal protein. Antibodies can be detected to the retinal protein within 7 days, and usually by ten days after immunization, inflammatory changes can be found involving the retina as well as other optical structures. Animals treated with

succinylacetone from the first day of retinal protein injection were totally protected from the development of inflammatory uveitis. Even if the inflammatory uveitis process was allowed to initially develop to the point that antibodies to the retinal protein had been produced, initiation of succinylacetone therapy at 7-8 days was still able to totally prevent the destructive retinitis associated with this animal model. This form of therapy is the most effective in this animal model, exceeding the effectiveness of cyclosporin A, the current agent of choice in the therapy of autoimmune disorders. The mechanism of action of succinylacetone is unclear. Although the agent has an antiproliferative effect to T cells and B cells in vitro, the dose required in vitro also inhibited the proliferation of non-lymphoid cells, such as fibroblasts and tumor cells. The observation that bone marrow engraftment proceeds normally in the presence of profoundly immunosuppressive effects also suggest that this is not a general antiproliferative type agent. Preliminary studies of adding various growth factors to cells in vivo and in vitro have been unable to overcome the immunosuppressive effects of the drug. We have extensively analyzed histology and blood chemistries in animals treated with toxic doses of succinylacetone and have found no consistent pathological finding other than a profound depletion of the lymphoid areas in all tissues. With 14 days of high-dose succinylacetone therapy, the thymus of treated animals almost totally involutes, giving the histological appearance of the thymus seen in infants with one of the forms of SCID. Thus, succinylacetone, in addition to being a profound and potentially useful clinical immunosuppressive drug, may provide us with a mechanism for studying a variety of cellular pathways.

Publications:

Kantoff PW, Gillio A, McLachlin JR, Bordignon C, Eglitis MA, Kernan NA, Moen RC, Kohn DB, Yu S-F, Karson E, Karlsson S, Zweibel JA, Gilboa E, Blaese M, Nienhuis AW, O'Reilly RJ, Anderson WF. Expression of human adenosine deaminase in non-human primates after retroviral mediated gene transfer. J Exp Med 1987;166:219-34.

Briles DE, Scott G, Gray B, Crain MJ, Blaese RM, Nahm M, Haber P, Scott V, Commer C. Naturally occurring anti-phosphocholine antibodies: A potential index of anti-polysaccharide responsiveness. J Infect Dis 1987;155:1307-14.

Hess RA, Tschudy DP, Blaese RM. Immunosuppression by succinylacetone II: Prevention of graft versus host disease. J Immunol 1987;139:2845-49.

Eglitis MA, Kantoff PW, McLachlin JR, Gillio A, Flake AW, Bordignon C, Moen RC, Karson EM, Zwiebel JA, Kohn DB, Gilboa E, Blaese RM, Harrison MR, Zanjani ED, O'Reilly R, Anderson WF. Gene therapy: Efforts at developing large animal models for autologous bone marrow transplant and gene transfer with retroviral vectors. CIBA Symposium No. 130, Molecular approaches to human polygenic diseases. 1987; 229-246.

Kohn DB, Kantoff PW, Eglitis MA, McLachlin JR, Moen RC, Karson E, Zwiebel JA, Nienhuis AW, Karlsson S, O'Reilly R, Gillio A, Bordignon C, Gilboa E, Zanjani ID, Blaese RM, Anderson WF. Retroviral-mediated gene transfer into mammalian cells. Blood Cells 1987;13:285-98.

Eglitis MA, Kantoff PW, Gillio A, Bordignon C, McLachlin JR, Moen RC, Kernan NA, Kohn DB, Yu S-F, Karlsson S, Karson EM, Zwiebel JA, Gilboa E, Blaese RM, Nienhuis A, O'Reilly RJ, Anderson WF. Expression of human adenosine deaminase in the hematopoietic cells of monkeys. In: Dicke KA, Spitzer G, Jagannath S, eds. Houston: Proc Third Int'l Symp on Autologous Bone Marrow Transplantation 1987;709-16.

Hess RA, Blaese RM, Tschudy DP. Succinylacetone as the sole immunosuppressive agent in total allogeneic bone marrow transplantation. In: Progress in bone marrow transplantation. Gale R, Champlin R, eds. NY: Alan R Liss, 1987;315-19.

Blaese RM, Kohn DB, Moen RC. The treatment of adenosine deaminase deficiency. Immunol Today 1987;8:296-7.

Wang F, Blaese RM, Zoon KC, Tosoto G. Suppressor T cell clones from patients with acute EBV induced infections mononucleosis. J Clin Invest 1987;79:7-14.

Kohn DB, Anderson W, Blaese RM. Gene therapy for genetic diseases. Cancer Investigation 1988, in press.

Eglitis MA, Kohn DB, Moen RC, Blaese RM, Anderson WF. Infection of human hematopoietic progenitor cells using a retroviral vector with a xenotropic pseudotype. Biochem Biophys Res Commun 1988;151:201-6.

Blaese RM, Lane EC. Primary immunodeficiency diseases and AIDS. In: Textbook of Rheumatology 1988, in press.

Blaese RM, Poplack DP, Muchmore AV. Disorders of the mononuclear phagocyte system. In: Immunologic Disorders of Infants and Children. 1988, in press.

Douglas S, Blaese RM. The mononuclear phagocytes. In: Immunologic Disorders of Infants and Children. 1988, in press.

Wright JJ, Birx DL, Wagner DK, Waldmann TA, Blaese RM, Fleisher TA. Normalization of antibody responsiveness in a patient with common variable hypogammaglobulinemia and HIV infection. N Engl J Med 1987;317:1516-20.

Blaese RM. Immunodeficiency syndromes with defects in both T and B cell functions. In: Metabolic Basis of Inherited Diseases. 1988, in press.

Skolik SA, Palestine AG, Blaese RM, Nusenblatt RB, Hess RA. Treatment of experimental autoimmune uveitis in the rat with systemic succinylacetone. Clin Immunol Immunopath. 1988, in press.

Kohn DB, Mitsuya H. Ballow M, Selegue JE, Barankiewicz J, Cohen A, Gelfand E, Anderson WF, Blaese RM. Establishment and characterization of adenosine deaminase (ADA) deficient human T cell lines. J Immunol 1988, in press.

Gillio A, Bordignon C, Kernan N, Kantoff P, Eglitis M, McLachlin J, Karson E, Yu S-F, Zwiebel J, Nienhuis A, Karlsson S, Blaese RM, Kohn D, Armentano D, Gilboa E, Anderson WF, O'Reilly RJ. Retroviral-mediated gene transfer and expression in

nonhuman primates following autologous bone marrow transplantation. In: Peschle C, ed. Normal and neoplastic blood cells: From genes to therapy. Ann NY Acad Sci 1987;511:406-17.

Eglitis MA, Kantoff PW, Kohn DB, Karson E, Moen RM, Lothrop CD Jr, Blaese RM, Anderson WF. Retroviral-mediated gene transfer into hematopoietic cells. In: Tavassoli, ed. Third Symp on Molecular Biology of Hemopoiesis. Plenum Press, 1988, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04016-15 MET

PERIOD COVERED
October 1, 1987 through September 30, 1988

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
	Mark Sklar	Medical Staff Fellow	MET, NCI
	Wieland Kiess	Guest Researcher	MET, NCI
	Matthew M. Rechler	Senior Investigator	NIADDK
	Barbara Linder	Medical Staff Fellow	NICHD

COOPERATING UNITS (if any)

LAB/BRANCH
Metabolism Branch

SECTION
Endocrinology Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	3	2

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors "B" 100%

☐ (a2) Interviews

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

Molecular cloning of the human type II IGF receptor by Rutter and his collaborators revealed an 80% sequence homology with the bovine cation independent mannose 6-phosphate receptor suggesting that this receptor is multifunctional, binding both IGF-II and lysosomal enzymes. We have performed biochemical experiments which support this proposal. Rat liver type II IGF receptor, purified by the conventional method of IGF-II affinity chromatography, bound quantitatively to a ß-galactosidase affinity column and was eluted with Man-6-P. Bovine liver Man-6-P receptor prepared using the conventional method of affinity chromatography on phosphomannan-Sepharose, bound IGF-II with high affinity (Kd=1nM). For immunologic studies, we purified type II IGF receptors and Man-6P receptors in parallel from rat placental membranes using either IGF-II-or ß-galactosidase affinity chromatography. A panel of 5 antisera that previously had been raised against either type II IGF receptor or Man-6-P receptor, behaved identically toward type II receptor versus Man-6-P receptor in ligand blocking and immunoprecipitation assays. We have performed additional experiments to define interactions of the two classes of ligands (IGFs and lysosomal enzymes) for binding to the IGF-II/Man-6-P receptor. We found that a panel of lysosomal enzymes inhibited the binding of ^{125}I-IGF-II to the purified receptor and that this inhibition resulted from a change in the binding affinity for IGF-II. We also examined the ability of IGF-II to modulate the binding of radiolabeled lysosomal enzyme (ß-galactosidase) to the receptor. IGF-II inhibited the binding of ^{125}I-ß-galactosidase to purified receptor and also inhibited the uptake of ^{125}I-ß-galactosidase in two cell lines in culture (C6 and BRL-3A) via the IGF-II/Man-6-P receptor. Additionally, we have examined the developmental pattern of IGF-II/Man-6-P receptor expression by extracting rat tissues and performing Western blotting using an antisera which is highly specific for the IGF-II/Man-6-P receptor. We found that the receptor is present in multiple tissues at levels as high as 1% of extractable protein and that the receptor is developmentally regulated, being high in fetal tissues and declining postnatally to much lower levels by day 20.

Project Description

Major Findings:

Biochemical evidence for identity of the type II IGF receptor and the mannose 6-phosphate receptor.

Molecular cloning of the human type II IGF receptor revealed an 80% sequence homology with the bovine mannose 6-phosphate receptor suggesting identify of the two receptors. We performed biochemical experiments which support this proposal. Rat liver type II IGF receptor, purified by the conventional method of IGF-II affinity chromatography, bound quantitatively to a ß-galactosidase affinity column and was eluted with Man-6-P. Bovine liver Man-6-P receptor, prepared by the conventional method of affinity chromatography on phosphomannan-Sepharose, bound IGF-II with high affinity (Kd=1 nM). Affinity crosslinking of ^{125}I-IGF-II to the Man-6-P receptor and analysis by SDS-gel electrophoresis, showed that ß-galactosidase, but not Man-6-P, inhibited the formation of the 250 kDa ^{125}I-IGF-II:receptor complex. The inhibition by ß-galactosidase was prevented by coincubation with Man-6-P. For immunologic studies we purified type II IGF receptors and Man-6-P receptors in parallel from rat placental membranes using either IGF-II or ß-galactosidase affinity chromatography. A panel of 5 antisera that previously had been raised against either type II IGF receptor or Man-6-P receptor, behaved identically toward type II IGF receptor versus Man-6-P receptor in ligand blocking and immunoprecipitation assays. These data support the conclusion that the type II IGF receptor and the cation-independent Man-6P receptor are the same protein and that the IGF-II and Man-6-P binding sites are distinct.

Inhibition of cellular uptake of the lysosomal enzyme ß-galactosidase by IGF-II.

To find out whether or not IGF-II could modulate the uptake of lysosomal enzymes via the IGF-II/Man-6-P receptor we first purified ^{125}I-ß-galactosidase on an IGF-II/Man-6-P affinity column to select for high affinity forms of ^{125}-ß-galactosidase. We utilized this preparation of ^{125}I-ß-galactosidase to study the uptake of the lysosomal enzyme by C_6 glial cells and BRL 3A2 cells in culture. An antibody to the IGF-II/Man-6-P receptor inhibited uptake. IGF-II inhibited uptake more effectively than IGF-I and insulin was ineffective. IGF-II also blocked the binding the ^{125}I-ß-galactosidase to highly purified IGF-II/Man-6-P receptor. These findings support the conclusion that the inhibition of the cellular uptake of ^{125}I-ß-galactosidase by IGF-II is occurring via the IGF-II/Man-6-P receptor and suggest that IGF-II can modulate the targeting of extracellular lysosomal enzymes to lysosomes.

Modulation of binding of ^{125}I-IGF-II to the IGF-II/Man-6-P receptor by lysosomal enzymes and Man-6-P.

Several lysosomal enzymes (ß-galactosidase, α-fucosidase, cathepsin D, and spingomylinase) inhibited the binding of 1^{25}I-IGF-II to highly purified IGF-II/Man-6-P receptor. Scatchard analysis of the binding data in the presence of a concentration of lysosomal enzyme that partially inhibited binding showed that the lysosomal enzymes decreased the binding affinity for IGF-II rather than decreasing the number of binding sites. Paradoxically, Man-6-P actually

increased the binding of 1^{25}I-IGF-II to some preparations of receptor. Scatchard analysis of the binding data revealed that Man-6-P increased the binding affinity for IGF-II. This effect of Man-6-P was greatest for membrane preparations, solubilized membranes, and receptors that had been purified on an IGF-II affinity column. IGF-II/Man-6-P receptors that had been purified on lysosomal enzyme affinity column displayed little if any Man-6-P effect. In any case, since lysosomal enzymes are present in extracellular fluids, they could modulate responses to IGF-II that are mediated by the IGF-II/Man-6-P receptor.

Developmental pattern of tissue IGF-II/Man-6-P receptor in the rat.

We used Western blotting with antiserum to the IGF-II/Man-6-P receptor to measure the levels of receptor in tissue extracts from fetal rats and early postnatal animals. By using a highly purified receptor standard and a combination of 1^{25}I-Protein A and immunoperoxidase staining we were able to cut out stained receptor bands from nitrocellulose paper, measure the radioactivity in a gamma counter, and quantitate the receptor levels as percent of extractable protein. We found that the levels of receptor were high in fetal tissue representing as much as 2% of extractable protein in the case of heart. The concentration of the receptor declined in all tissues postnatally. By extracting whole embryos we were able to demonstrate significant levels of receptor at at the earliest time examined (6 days gestation). These data suggest that the IGF-II/Man-6-P receptor may play a role in fetal growth and/or development.

The rat C_6 glial cell line as a model to study the role of insulin-like growth factors in supporting cells of the CNS.

Insulin-like growth factors are thought to play a role in the growth and development of the central nervous system. To explore the role of glial cells (astrocytes and oligodendrocytes) in this process, we choose a rat glial cell line C_6 which has retained some glial cell properties. We found that this cell line produces IGF-I but not IGF-II. This was confirmed by the measurement of mRNA by Northern blotting which showed the presence of IGF-I transcripts but no IGF-II mRNA. We identified large numbers of IGF-II/Man-6-P receptors on these cells but very few IGF-I receptors and no insulin receptors. Exposure of the cells to an antibody to the IGF-II/Man-6-P receptor completely blocked the uptake and degradation of IGF-II introduced into the medium. Although the C_6 glial cells produced IGF-I we were unable to demonstrate that the secreted IGF-I played a role in autocrine growth control for these cells. Our data do suggest that glial cells in the CNS could produce IGF-I which could play a paracrine role in the growth and differentiation of neural cells. In addition, IGF-II produced elsewhere (choroid plexus) could modulate the function of IGF-II/Man-6-P receptors on glial cells to target lysosomal enzymes to lysosomes and glial cells could serve to modulate IGF-II functions by removing it from the intercellular spaces.

Biosynthesis of the IGF-II/Man-6-P receptor in C_6 glial cells.

We confirmed the biosynthetic pathway for the IGF-II/Man-6-P receptor in C_6 cells that had been described by others in other cell lines. In addition we demonstrated with the use of tunicamycin, an inhibitor of the earliest step in N-linked glycosylation, that receptor that was devoid of N-linked sugars was still

able to bind IGF-II. Also, purified receptor that was stripped of N-linked carbohydrate by treatment with N-glycanase showed normal binding affinity for IGF-II. These results indicate that the carbohydrate structure of the receptor is not essential for ligand binding and are consistent with the possibility that IGF-II could inhibit binding of lysosomal enzymes to the receptor early in its transit through the Golgi stack.

Publications:

Rechler MM, Nissley SP, Roth J. Hormonal regulation of human growth. [Editorial]. New Engl J of Med 1987;316:941-3.

Romanus JJ, Yang Y W-H, Nissley SP, Rechler MM. Biosynthesis of the low molecular weight carrier protein for insulin-like growth factors in rat liver and fibroblasts. Endocrinol 1987;121:1041-50.

Kiess W, Haskell JF, Lee L, Greenstein LA, Miller BE, Aarons AL, Rechler MM, Nissley SP. An antibody that blocks insulin-like growth factor (IGF) binding to the type II IGF receptor is neither an agonist nor an inhibitor of IGF-stimulated biologic responses in L6 myoblasts. J Biol Chem 1987;262:12745-51.

Kiess W, Greenstein LA, White RM, Lee L, Rechler MM, Nissley SP. The type II insulin-like growth factor receptor is present in rat serum. Proc Natl Acad Sci USA 1987;84:7720-4.

Kozak RW, Haskell JF, Greenstein LA, Rechler MM, Waldmann TA, Nissley SP. Type I and II insulin-like growth factor receptors on human phytohemagglutinin-activated T lymphocytes. Cell Immunol 1987;109:318-31.

Hill JM, Lesniak MA, Kiess W, Nissley SP. Radioimmunohistochemical localization of type II IGF receptors in rat brain. Peptides 1988, in press.

Rechler MM, Yang Y W-H, Brown AL, Romanus JA, Adams SO, Kiess W, Nissley SP. Insulin-like growth factors in fetal growth and cell development. Intl Symposium on Growth Hormones: Basic and Clinical Aspects 1987, in press.

Tseng, L Y-H, Schwartz GP, Sheikh M, Chen ZZ, Hoshi S, Wang J-F, Nissley SP, Burke GT, Katsoyannis PG, Rechler MM. Hybrid moleulces containing the A-domain of insulin-like growth factor-I and the B chain of insulin have increased mitogenic activity relative to insulin. Biochem Biophys Res Comm 1987;149:672-79.

Rechler MM, Nissley SP. Molecular studies of insulin-like growth factor II biosynthesis and action. In: Isaksson O, Binder C, Hall K, Hockfelt B, eds. Nordisk Insulin Symposium 1: Growth Hormone, Basic and Clinical Aspects. New York, Elsevier Biomed Press ICS 748, Exerpta Medica 1987;425-39.

Romanus JA, Yang Y W-H, Adams So, Sofair AN, Tseng L Y-H, Nissley SP, Rechler MM. Synthesis of insulin-like growth factor II (IGF-II) in fetal rat tissues: translation of IGF-II ribonucleic acid and processing of pre-pro-IGF-II. Endocrinol 1988;122:709-16.

Kiess W, Blickenstaff GD, Sklar MM, Thomas CL, Nissley SP, Sahagian GG. Biochemical evidence that the type II insulin-like growth factor receptor is identical to the cation-independent mannose 6-phosphate receptor. J Biol Chem 1988, in press.

Nissley SP. Growth factors. In: Becker KL, ed. Principles and Practice of Endocrinology and Metabolism. Lippencott 1988, in press.

Gelato MC, Kiess W, Lee L, Malozowski S, Rechler MM, Nissley SP. The insulin-like growth factor-II/mannose 6-phosphate receptor is present in monkey serum. J Clin Endocrinol Metab 1988, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04017-10 MET

PERIOD COVERED

October 1, 1987 through September 30, 1988

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)*

PI:	David L. Nelson	Head, Immunophysiology Section	MET, NCI
	William D. Schwieterman	Medical Staff Fellow	MET, NCI
	Luisa Marcon	Visiting Fellow	MET, NCI
	Luigi D. Notarangelo	Guest Researcher	MET, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch

SECTION

Immunophysiology

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	4	0

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors "B" 100%

☒ (a2) Interviews

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

Studies were performed to examine the maturation and regulaton of the human immune response in normal individuals and in patients with congenital and acquired immune deficiency states associated with a high frequency of cancer. The interaction of the T-cell derived lymphokine interleukin-2 (IL-2) with its cell membrane receptor (IL-2R) plays a pivotal role in the establishment and maturation of the immune response. Soluble Tac protein from the human IL-2R is released from activated T-cells, B-cells, and monocytes in vitro and is also constitutively produced by leukemic cells in vitro. This soluble Tac protein is 10 kDa smaller than the Tac protein expressed on cell surfaces and specifically binds IL-2 with low affinity (20 nM). Soluble Tac protein was measurable by a double epitope enzyme-linked immunoassay (ELISA) in the serum and urine but not cerebrospinal fluid of all normal individuals. Elevated levels of soluble Tac protein were found in the serum of several retroviral associated diseases including the adult T-cell leukemia (ATL), hairy cell leukemia (HCL), and the acquired immune deficiency syndrome (AIDS). Favorable responses to therapy in ATL and HCL were associated with reductions in serum Tac protein. Among patients with non-Hodgkin's lymphoma, the serum level of Tac protein at the time of diagnosis was the best predictor of survival following therapy. Serum Tac protein was also elevated at the time of rejection episodes in liver and heart-lung transplant recipients and in patients with active sarcoidosis. Measurement of Tac protein in serum is useful in the diagnosis and management of certain cancer patients and in monitoring the state of immunologic activation in humans in vivo.

GPO 914-918

Project Description

Major Findings:

The cell membrane receptor for the T-cell derived lymphokine, interleukin-2 (IL-2) is a multichain structure consisting of at least two proteins with molecular weights of 55 and 75 kilodaltons (kDa). Hybridoma derived monoclonal antibodies binding to distinct epitopes on the 55 kDa molecule have been produced and this chain has been termed the Tac protein. The Tac molecule is released by activated normal T-cells, B-cells, and monocytes in vitro. In addition, several lymphoid tumor cell lines constitutively release large amounts of soluble Tac protein in vitro. The soluble Tac protein is 10 kDa smaller in size than the cell-membrane form of this molecule and specifically binds IL-2, albeit at low affinity (20 nM).

Using two monoclonal antibodies directed against distinct epitopes on the Tac protein, we constructed a "sandwich" enzyme-linked immunoassay (ELISA) to quantitatively measure soluble Tac molecules. Soluble Tac protein was measurable in the serum and urine but not cerebrospinal fluid of all normal individuals. Since soluble Tac protein was produced by activated normal lymphocytes and monocytes as well as constitutively produced by certain tumor cell lines, we undertook studies to measure the level of Tac protein in body fluids of certain patients with cancer and other patients whose immune system might be undergoing activation in vivo.

Elevated levels of soluble Tac protein were observed in diseases associated with human retroviral infections including the adult T-cell leukemia (ATL), hairy cell leukemia (HCL), and the acquired immune deficiency syndrome (AIDS). Elevated levels of Tac protein were also found in the urine of ATL patients and in the CSF from ATL patients with leukemic central nervous system disease. Favorable clinical responses to chemotherapy in ATL and recombinant alpha interferon therapy in HCL were associated with reductions in the serum levels of Tac protein. Treatment of ATL patients in vivo with antibodies to the Tac protein inhibit the "sandwich" ELISA and anti-mouse immunoglobulin responses in the sera of treated patients give "false positive" results in the same assay. Therefore a competitive ELISA was developed which is not influenced by either of these factors and was used to monitor anti-Tac therapy in ATL patients.

Among patients with non-Hodgkin's lymphoma, the level of Tac protein at the time of diagnosis was the single best available predictor of long term survival after chemotherapy by distinguishing those with an 85% versus those with a 35% chance of survival. The levels of soluble Tac protein were elevated in the serum and lung-wash fluids of patients with sarcoidosis, another disease of suspected retroviral etiology. In sarcoidosis patients, responses to corticosteroid therapy were followed by reductions in the serum level of Tac protein and this assay provided an excellent non-invasive method of following disease activity.

Among patients with rheumatoid arthritis, serum levels of Tac protein were significantly higher than normal and did not correlate with disease activity. Joint fluid Tac protein levels were higher than paired serum specimens suggesting local production by activated lymphoid cells in the joint tissues. Among recipients of allogeneic liver transplants and allogeneic heart-lung

transplants, elevations of soluble Tac protein were indicative of rejection episodes and poor outcome.

The measurement of Tac protein from the human IL-2 receptor in various body fluids is useful in the diagnosis and management of patients with neoplastic and inflammatory disorders.

Publications:

Boutin B, Wagner DK, Nelson DL. Analysis of CD3 antigen expression in patients with ataxia-telangiectasia. Clin and Exp Immunol 1987;68:320-30.

Wagner DK, Kiwanuka J, Edwards BK, Rubin LA, Nelson DL, Magrath IT. Soluble interleukin-2 receptor levels in patients with undifferentiated and lymphoblastic lymphomas: Correlation with survival. J Clin Oncol 1987;5:1262-74.

Nelson DL, Waldmann TA. Summary of the A1 Workshop. In: Leukocyte Typing III. McMichael A, et al, eds. Oxford: Oxford Univ Press 1987;525-26.

Nelson DL, Crisman J, Casabo L, Kurman CC. Analysis of anti-CD25 monoclonal antibodies by inhibition of soluble interleukin-2 receptor measurement by ELISA. In: Leukocyte Typing III. McMichael A, et al, eds. Oxford: Oxford Univ Press 1987;539-41.

Rubin LA, Hoekzema GS, Nelson DL, Greene WC, Jay G. Reconstitution of a functional interleukin receptor in a non-lymphoid cell. J Immunol 1987;139:2355-60.

Kloster B, John PA, Miller L, Rubin LA, Nelson DL, Blair DC, Tomar RH. Soluble interleukin-2 receptors are elevated in patients with AIDS or at risk of developing AIDS. Clin Immunol and Immunopathol 1987;45:440-46.

Boutin B, Matsuguchi L, Lebon P, Ponsot G, Nelson DL. Soluble IL-2 receptors in acute and subacute encephalitis. Ann Neurol 1987;22-658-61.

Lawrence EC, Berger MB, Brousseau KP, Rodriquez TM Siegel SJ, Kurman CC, Nelson DL. Elevated serum levels of soluble interleukin-2 receptors in active pulmonary sarcoidosis: relative specificity and association with hypercalcemia. Sarcoidosis 1987;4:87-93.

Hakimi J, Seals C, Anderson LE, Podlaski FJ, Lin P, Wahleed D, Jenson JC, Donadio PE, Familletti PC, Pan YE, Tslen W, Chizzonite RA, Casabo L, Nelson DL, Cullen BR. Biochemical and functional analysis of soluble human interleukin-2 receptor produced in rodent cells. Solid phase reconstitution of a receptor-ligand binding reaction. J Biol Chem 1987;262:17336-41.

Nelson DL, Wagner DK, Marcon L, Kurman CC, Fritz ME, Crisman JM, Casabo L, Rubin LA. An analysis of soluble interleukin-2 receptors in human neoplastic disorders. In: Albertini A, Lenfant C, Paoletti R eds. Biotechnology in Clinical Medicine. NY: Raven Press 1987;277-86.

Tomkinsin BD, Wagner DK, Nelson DL, Sullivan JL. Activated lymphocytes during acute Epstein Barr virus infection. J Immunol 1987;139:3802-7.

Goldstein AM, Marcon L, Cullen BR, Nelson DL. A competitive enzyme-linked immunoassay (ELISA) for the measurement of soluble human interleukin-2 receptors (IL-2R, Tac protein). J Immunol Methods 1988;107:103-9.

Lawrence EC, Brousseau KP, Kurman CC, Marcon L, Nelson DL. Elevated levels of soluble interleukin-2 receptors in serum and bronchoalverolar lavage fluids in active sarcoidosis. Am Rev Resp Dis 1988;137:759-64.

Scollard DM, Wagner DK, Nelson DL. Release of soluble interleukin-2 receptors (Tac peptide) in vivo during human immune responses to tuberculin. Clin Immunol Immunopathol 1988;46:450-5.

Yarchoan R, Strober W, Murphy BR, Nelson DL: In vitro production of IgA antibody by human lymphocytes. In: Strober W, Lamm ME, McGhee JR, James SP, eds. Mucosal immunity and infections at mucosal surfaces. Oxford: Oxford Univ Press 1988;94-103.

Steis RG, Marcon L, Clark J, Urba W, Longo DL, Nelson DL, Maluish AE. Serum soluble IL-2 receptor as a tumor marker in patients with hairy cell leukemia. Blood 1988;71:1304-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04018-12 MET

PERIOD COVERED
October 1, 1987 through September 30, 1988

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

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

PI:	Andrew V. Muchmore, M.D.	Senior Investigator	MET, NCI
	Bibhuti Mishra, M.D.	Clinical Associate	MET, NCI
	Neeraja Sathymoorthy, Ph.D.	Visiting Fellow	MET, NCI
	Jean Decker, B.S.	Chemist	MET, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Metabolism Branch

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	4	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors "B" 100%

☐ (a2) Interviews

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

Last year represented a transition in the major focus of my laboratory. Research has progressed from the purification, immunologic characterization, sequencing, and cloning of uromodulin and 85 KD immunorepressive glycoprotein derived from pregnancy urine to now focusing on the characterization of glycosylation patterns responsible for the biologic activity of uromodulin. Utilizing a combination of protease digestion and multiple chromatographic steps, we have purified to homogeneity and structurally characterized by ^1H NMR two profoundly biologically active high mannose oligosacharides. Based on these structural observations, alternative sources for active oligosaccharides have been found and include egg white protein, soybean meal, and yeast mannan. These compounds inhibit and enhance the immune response both in vitro and in vivo. Utilizing defined oligosaccharides ranging from 7-11 sugar residues, we have performed a number of structure function studies. These compounds inhibit T cell responsiveness, enhance PGE_2 synthesis, induce collagenase release, block hydrogen peroxide formation, activate the hexose monophosphate shunt, bind to IL-1, TNF, and IL-2, and regulate the expression of DR. We have applied for a patent covering the immunoregulatory activity of these structurally unique compounds.

PHS 6349 (Rev. 1/84) GPO 914-918

Project Description

Major Findings

1. The immunosuppressive oligosaccharides of uromodulin have been purified and structurally characterized.

2. A panel of related oligosaccharides have also been purified and structurally characterized.

3. These compounds have been shown to

   a. block T cell responsiveness in vitro.

   b. induce a delayed inflamatory response in vitro.

   c. induce PGE_2 synthesis in vivo.

   d. Compete with binding to IL-1, IL-2 and TNF.

We have also shown that IL-2 and TNF directly bind to Class II antigens and can regulate their expression in vitro. This binding is mediated by oligosaccharides expressed by Class II molecules.

Publications

Hession C, Decker JM, Sherblom AP, Kumar S, Yue CC, Mattaliano R, Tizard R, Kawashima ES, Chmeissner U, Heletky S, Chan P, Burne C, Show A, Muchmore AW. Uromodulin Tomm-Horstall glycoprotein: A renal ligand for lymphokines. Science 1987;237:1479-84.

Sherblom AP, Decker JM, Muchmore AV. The lectin-like interaction between recombinant tumor necrosis factor and uromodulin. J Biol Chem 1988;263:5418-24.

Blaese RM, Poplack DP, Muchmore AV. Disorders of the mononuclear phogocyte system. In: Immunologic Disorders of Infants and Children 1988, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04020-11 MET

PERIOD COVERED
October 1, 1987 - September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Antigen-Specific T-cell activation, and application to vaccines for malaria and AIDS

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

PI:	Jay A. Berzofsky, M.D., Ph.D.	Section Chief	MET, NCI
	Paula Hale, M.D.	Medical Staff Fellow	MET, NCI
	Akihiko Kurata, M.D., Ph.D.	Guest Researcher	MET, NCI
	Hidemi Takahashi, M.D., Ph.D.	Visiting Fellow	MET, NCI
	Anne Hosmalin, M.D.	Visiting Fellow	MET, NCI
	Rose Toper, Ph.D.	Visiting Fellow	MET, NCI
	Judy Kim	Hughes Medical Student	MET, NCI

See attached sheet

COOPERATING UNITS (if any)

Louis H. Miller	Chief, Malaria Sec.	LPD, NIAID	
Gene H. Shearer, Ph.D.	Section Chief	IB, NIAID	See
Robert Gallo, M.D.	Lab Chief	LTCB, NCI	Attached

LAB/BRANCH
Metabolism Branch

SECTION
Molecular Immunogenetics and Vaccine Research Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
11.5	9.5	2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

"B" 100%

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

T lymphocytes recognize a limited number of antigenic sites on any given antigenic protein. We find that the presence or absence of a response to one immunodominant site can make the difference between a high responder and a low responder, even though low responders respond to other sites almost as well as high responders. Besides interaction with major histocompatibility (MHC) molecules, we find that the mode by which the antigen is processed into fragments for T-cell recognition also determines which sites are seen. The products of natural processing of the protein appear to be larger than the synthetic peptides and contain structures which hinder binding to certain MHC molecules or to the T-cell receptor. Besides MHC binding and antigen processing, a third factor in immunodominance is the intrinsic structure of the antigenic site. We have previously shown that amphipathic helices have a high chance of being immunodominant, and have developed a computer program to locate such structures in protein sequences. We prospectively predicted sites in the malaria circumsporozoite protein and found that the four most widely recognized sites in an endemic area of West Africa were all predicted. Similarly, we identified two helper T-cell sites from the HIV (AIDS virus) envelope, and showed that immunization with these elicits enhanced antibody responses to the whole envelope when injected into monkeys. These sites are also recognized by human T cells from volunteers who have been immunized with a recombinant vaccinia virus expressing the HIV envelope. In mice, we have now identified other immunodominant sites in the envelope protein, including a very large one within which different MHC types of mice each recognize different overlapping regions. Also, since cytotoxic T lymphocytes (CTL) may play a critical role in defense against AIDS, we have used a recombinant vaccinia virus and transfectants expressing the HIV envelope gene to induce specific CTL against the HIV envelope. Using synthetic peptides, we were able to identify the first known CTL recognition site in the AIDS virus. However, we observed that such sites are very limited. These results may hopefully contribute to the design of a vaccine for AIDS.

Continuation Sheet for PHS 6040

Other Professional Personnel:

Michael Good, M.D., Ph.D.	Guest Researcher	Met, NCI;LPD, NIAID
Hanah Margalit, Ph.D.	Visiting Fellow	LMB,NCI

Other Cooperating Units:

Ronald N. Germain, M.D., Ph.D.	Section Chief	LI, NAID
Bernard Moss, M.D., Ph.D.	Lab Chief	LVD, NIAID
Scott Durum, Ph.D.	Senior Investigator	BRMP,FCRF,NCI

Project Description

Major Findings

We studied the frequencies of proliferating T cells specific for myoglobin and specific epitopes thereof and restricted to Major Histocompatibility (MHC) molecules from high and low responder haplotypes, using limiting dilution Poisson analysis. The 3-4-fold difference between high and low responder types could be accounted for almost completely by the response to an immunodominant epitope (102-118) representing two thirds of the high responder population but absent in the low responder population. The low responder also failed to use the I-A molecule, with which the response to the immunodominant site was associated, but used only the I-E molecule. Thus, the response to a single immunodominant site can determine high or low responsiveness to the whole protein. The T cells specific for other sites were almost equally frequent in the high and low responder populations, but these never compensated in the low responder for the absence of a response to the immunodominant site. Thus there must be something unique about immunodominant antigenic sites.

We found that one feature that determines responsiveness to a given epitope is the mode of antigen processing of the protein. By immunizing with peptides, we could demonstrate the ability of a specific peptide to bind to the MHC molecules and the presence of T cells that could recognize such a complex, but no such response was detected when immunizing with the native protein. Moreover, the T-cells specific for this peptide did not crossreact with the native protein. Yet in another strain differing only in MHC, immunization with either the peptide or native protein gave T cells equally reactive to both. Studies with F_1 hybrid presenting cells showed that the results were not due to MHC-linked processing differences. Similarly, a T-cell clone not crossreactive between two species of native myoglobins were very crossreactive at the peptide level. Therefore, the lack of crossreactivity was due to structural features outside the antigenic site itself. The best explanation for all these data appears to be that the products of natural processing of the protein are larger than the synthetic peptides and contain structures which hinder binding to the relevant MHC molecules or to the T-cell receptor.

We have previously shown that amphipathic helices have a higher than random chance of being immunodominant, and have developed a computer program to locate such structures in protein amino acid sequences. We prospectively predicted sites in the malaria circumsporozoite protein and then tested overlapping peptides covering the whole molecule for their ability to elicit proliferation of T-cells from patients in an endemic area of West Africa, in collaboration with Drs. Michael Good and Louis Miller of the Malaria Section, LPD, NIAID. We found that the four most widely recognized sites were all predicted, and that only one strongly predicted site was not seen by this population. However, the immunodominant sites were all from regions of the protein that vary from strain to strain. For one major site, we narrowed down the site necessary for T-cell help and proliferation in two MHC types of mice. The epitopes recognized by the two types of mice overlapped, but both contained variable amino acids. Therefore, the parasite has discovered the T-cell epitopes before us and has learned to vary them to escape immune pressure.

We have shown that immunization with antigenic peptides of the malaria circumsporozoite protein mixed with IL-2 in the adjuvant enhances response in genetic low responder animals. This effect depends on the biologic activity of the IL-2, because a mutant IL-2 that retains immunogenicity but lack lymphokine activity fails to enhance the response. The mechanism appears to involve enhancement of B-cell responses in the presence of limiting amounts of help. This may be a useful method of immunopotentiation in vaccine development.

We have used the amphipathicity approach to predict helper T-cell sites in the envelope of the AIDS virus, HIV. These were first demonstrated in mice, and one of these can elicit immunity to the native envelope protein in three of four different MHC types of mice. We have now immunized rhesus monkeys with these peptides to determine whether they would prime helper T cells and so enhance antibody responses on subsequent challenge with the whole envelope protein. The animals that received peptide first made greatly enhanced antibody responses compared to the monkeys that received the protein alone. Thus these peptides are recognized and induce help in primates and may therefore be useful components of a vaccine.

We have tested the response to these peptides in the peripheral blood lymphocytes of human volunteers immunized by Dr. Daniel Zagury with a recombinant vaccinia virus expressing the gene for the HIV envelope protein. Of the eleven who had been boosted within six weeks of the test, 8 gave T-cell responses to one of the peptides, env T1, residues 428-443. Thus, this peptide is also seen by a significant fraction of immune humans of diverse HLA types.

We have made a larger series of synthetic peptides covering about half of the envelope protein of HIV, in collaboration with Dr. Richard Houghten (Scripps). We have used these to define other helper T-cell sites recognized by mice of four different MHC haplotypes. One such region we identified contains overlapping epitopes that are seen by the 4 MHC types differently, but all recognize the same cluster. Thus, this region of the envelope may be especially important as a vaccine component, and may overcome MHC restriction problems.

We have identified the first cytotoxic T-cell (CTL) epitope from any protein of the AIDS virus. In collaboration with Drs. Bernard Moss and Ronald Germain, NIAID, we have used a recombinant vaccinia virus and transfectants expressing the HIV envelope gene to induce specific CTL against HIV envelope in mice. These were conventional CD8, class I-MHC molecule restricted CTL and both their induction and their killing activity were specific for cells expressing the envelope protein. Using synthetic peptides, we were able to identify the first known CTL recognition site in the AIDS virus. This appears to be the only immunodominant site of 41 peptides tested. Thus, such sites are very limited. Also, of 5 different class I MHC molecules that could have presented HIV envelope sites in two different MHC types, only one (D^a) appeared to be used. This site, residues 315-329, is highly variable among HIV isolates, and coincidentally, corresponds to the major neutralizing antibody epitope identified by three groups. Thus, the variability of the site may be induced by immune pressure from either antibodies or CTL. As virus can spread from cell to cell without being free in solution and accessible to antibodies, the CTL may be very important in protection against AIDS.

We developed a way to fuse existing T-cell clones to produce hybridomas. Thus we were able to compare matched pairs of hybridomas with the corresponding nontransformed clones. They were similar in specificity and many properties, but the clones were better at killing and the hybridomas at IL-2 production. The most striking difference was that the hybridomas consistently lost the clones' ability to make IL-1. Thus, hybridomas are not perfect models of normal T cells.

Publications:

DeLisi C, Cornette J, Margalit H, Cease K, Spouge J, Berzofsky JA. The role of amphipathicity as an indicator of T cell antigenic sites on proteins. In: Sercarz E, Berzofsky JA, eds. Immunogenicity of Protein Antigens: Repertoire and Regulation. Vol I, Boca Raton, CRC Press 1987;35-42.

Berzofsky JA, Cease KB, Berkower IJ, Margalit H, Cornette J, Spouge J, Spencer C, Buckenmeyer G, Streicher H, Kojima M, Delisi C. The role of MHC and amphipathic structures in T cell recognition: Features determining immunodominance. In: Vogel H, Pernis B, Silverstein S, eds. Antigen Processing and Presentation, NY, Columbia Univ Press 1988;125-31.

Kawamura H, Berzofsky JA. Influence of MHC-linked genes on idiotype expression. In: Sercarz E, Berzofsky JA, eds. Immunogenicity of Protein Antigens: Repertoire and Regulation. Vol II, Boca Raton, CRC Press 1987;121-27.

Berzofsky JA, Cease KB, Ozaki S, Margalit H, Cornette JL, Spouge J, DeLisi C: Helper T cell immunity: Implications for vaccines. In: Chanock R, Brown F, Ginsberg H, Lerner R, eds. Vaccines '87. Cold Spring Harbor, NY: Cold Spring Harbor Lab 1987;26-32.

Good MF, Berzofsky JA, Maloy L, Lunde MN, Hayashi Y, Fujii N, Hockmeyer WT, Moss B, Miller LH. Genetic control of the immune response to a plasmodium falciparum sporozoite vaccine and to the Cirumsporozoite [CS] protein. In: Chanock R, Brown F, Ginsberg H, Lerner R, eds. Vaccines '87. Cold Spring Harbor, NY: Cold Spring Harbor Lab 1987;81-85.

Hoffman SL, Cannon LT, Berzofsky JA, Majarian WR, Young JG, Maloy WL, Hockmeyer WT. Plasmodium falciparum: Increased immunity with T cell epitope on sporozoite vaccine. Exp Parasitol 1987;64:64-70.

Berzofsky JA, Cease KB, Cornette JL, Spouge JL, Margalit H, Berkower IJ, Good MF, Miller LH, DeLisi C. Protein antigenic structures recognized by T cells: Potential applications to vaccine design. Immunol Rev 1987;98:9-52.

Berzofsky JA. Features of T-cell recognition and antigen structure useful in the design of vaccines to elicit T-cell immunity. Vaccines 1988;6:89-93.

Berzofsky JA, Ozaki S. Antibody conjugates mimic specific B-cell presentation of antigen: epitope restrictions in specific T-B interaction. In: Schook LB, Tew JG, eds. Antigen presenting Cells: Diversity, Differentiation and Regulation. NY, Alan R Liss 1988;41-8.

Good MF, Pombo D, Quakyi IA, Riley EM, Houghten RA, Menon A, Alling DW, Berzofsky JA, Miller LH. Human T cell recognition of the circumsporozoite protein of Plasmodium falciparum. Immunodominant T cell domains map to the polymorphic regions of the molecule. Proc Natl Acad Sci USA 1987;85:1199-1203.

Good MF, Berzofsky JA, Miller LH. The T-cell response to the malaria circumsporozoite protein: an immunological approach to vaccine development. Ann Rev Immunol 1988;6:663-88.
Lin J, Berzofsky JA, Delovitch TL. Ultrastructural study of internalization and recycling of antigen by antigen presenting cells. J Mol Cell Immunol 1988, in press.

Kojima M, Cease KB, Buckenmeyer GK, Berzofsky JA. Limiting dilution comparison of the repertoires of high and low responder MHC-restricted T cells. J Exp Med 1988;167:1100-13.

Good MF, Pombo D, Maloy WL, de La Cruz VF, Miller LH, Berzofsky JA. Parasite polymorphism present within minimal T-cell epitopes of Plasmodium falciparum circumsporozite protein. J Immunol 1988;140:1645-50.

Takahashi H, Cohen J, Hosmalin A, Cease KB, Houghten R, Cornette J, DeLisi C, Moss B, Germain RN, Berzofsky JA. An immunodominant epitope of the HIV gp160 envelope glycoprotein recognized by class I MHC molecule-restricted murine cytotoxic T lymphocytes. Proc Natl Acad Sci USA 1988;85:3105-9.

Darst SA, Robertson CR, Berzofsky JA. Adsorption of the protein antigen myoglobin affects the binding of conformation-specific monoclonal antibodies. Biophys J 1988, in press.

Pombo D, Maloy WL, Berzofsky JA, Good MF. Neonatal exposure to immunogenic peptides. Differential susceptibility to tolerance induction of helper T cells and B cells reactive to malarial circumsporozoite peptide epitopes. J Immunol 1988;3594-3598.

Berzofsky JA. T-cell recognition of protein and peptide antigens: applications to vaccine development In: Laskey L, ed. Technological Advances in Vaccine Development. NY, Alan R Liss 1988, in press.

Berzofsky JA. Immunodominance in T lymphocyte recognition. Immunol Ltrs 1988, in press.

Ozaki S, Durum SK, Muegge K, York-Jolley J, Berzofsky JA. Production of T-T hybrids from T cell clones: Direct comparison between cloned T cells and T hybridoma cells derived from them. J Immunol 1988, in press.

Brett SJ, Cease KB, Berzofsky JA. Influences of antigen processing on the expression of the T cell repertoire: Evidence for MHC-specific hindering structures on the products of processing. J Exp Med 1988, in press.

Good MF, Pombo D, Lunde MN, Maloy WL, Halenbeck R, Koths K, Miller LH, Berzofsky JA. Recombinant human interleukin-2 (IL-2) overcomes genetic non-responsiveness to malaria sporozoite peptides. Correlation of effect with biological activity

of IL-2. J Immunol 141, 1988, in press.

PATENT:

Berzofsky JA, Takahashi H, Hosmalin A, Germain RN, Moss B. US Patent 4/148,692: A synthetic antigen evoking anti-HIV response, January 26, 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB-04024-1

PERIOD COVERED
October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Control of gene expression in lymphoid development

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

P.I.:	Louis M. Staudt, M.D.	Senior Staff Fellow	MET, NCI
	Patricia Fast, M.D.	Medical Staff Fellow	MET, NCI
	Hon-Sum Ko, M.D.	Fogarty Visiting Fellow	MET, NCI

COOPERATING UNITS (if any)

	Wesley McBride	Senior Investigator	LB, NCI

LAB/BRANCH
Metabolism Branch

SECTION

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	3	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors "B" 100%

☐ (a2) Interviews

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

As an approach to understanding development in the B lymphocyte lineage, we have characterized trans-acting factors that mediate lymphoid-specific gene transcription. The promoters of all immunoglobulin (Ig) variable genes and the Ig heavy chain enhancer contain a critical octamer DNA motif, ATTTGCAT. We demonstrated that this motif, together with a TATA motif, is necessary and sufficient for lymphoid-specific promoter activity. We then assayed nuclear extracts of lymphoid and non-lymphoid cell lines for proteins which specifically bound to the octamer motif and detected two major species: a ubiquitous octamer binding protein (NF-A1) and a lymphoid-specific octamer binding protein (NF-A2). We hypothesized that NF-A2 mediated the lymphoid-specific activity of Ig promoters. We have cloned a gene which most likely encodes the human NF-A2 gene by screening a phage lambda gt11 expression library with a radiolabelled DNA probe containing the octamer motif. The cloned gene, termed oct-2, encodes an octamer-specific DNA binding protein which is lymphoid-restricted in its expression. This is the first gene to be cloned that encodes a tissue-specific DNA binding protein. The oct-2 gene sequence revealed a 60 amino acid domain that is homologous to the "homeo box" domain of proteins which are important for cell type determination and development in yeast and Drosophila. This domain of the oct-2 protein was shown to be required for DNA binding by site directed mutagenesis. This represents the first demonstration that a mammalian homeo box gene encodes a sequence-specific DNA binding protein. The presence of the homeo box in the oct-2 gene lends support to the notion that this gene is important for lymphoid development. Recent evidence indicates that the octamer motif may control the expression of other lymphoid-specific genes besides Ig, notably the BCL-2 gene involved in the pathogenesis of human follicular lymphomas. Using the cloned oct-2 gene we will be able to determine the range of genes which are expressed exclusively in lymphocytes under the transcriptional control of the octamer motif.

GPO 914-918

Project Description

Major Findings:

Cloning of a gene (oct-2) which encodes a lymphoid-restricted protein that binds to the immunoglobulin octamer DNA motif.

Immunoglobulin (Ig) genes are expressed only in lymphoid cells and this expression depends on a regulatory octamer DNA motif, ATTTGCAT, found in Ig promoters and in the Ig heavy chain enhancer. At least two nuclear proteins bind the octamer motif specifically: NF-A1, which is found in all cell types, and NF-A2, which is restricted to lymphoid cells. NF-A2 most likely mediates the lymphoid-specific transcriptional activity of Ig promoters. In order to understand this transcriptional activation on a molecular level we decided to clone the gene encoding NF-A2. We used a new method of screening expression libraries in phage lambda gt11 with radiolabelled DNA probes containing the octamer motif. An important modification of the protocol made the cloning possible: DNA probes were used that were tandem repeats of the octamer motif. These multimer probes gave readily detectable signals from phage plaques that could not be detected using probes containing only one copy of the octamer motif. The two recombinant phage isolated by this procedure contained cDNA inserts encoding proteins that bound specifically to the octamer motif. The cDNA inserts of these two phage cross-hybridized with each other and define what we term the oct-2 gene. The DNA binding characteristics of the oct-2 gene product and NF-A2 were virtually identical. Furthermore, oct-2 was found to be expressed predominantly in lymphoid cells. The amount of oct-2 mRNA expressed in a given lymphoid cell line correlated well with the amount of NF-A2 found in the same cell line. Pre-B cell lines showed a coordinate increase in oct-2 mRNA and NF-A2 protein upon stimulation with bacterial lipopolysaccharide. Given these correlations, the oct-2 gene most likely encodes NF-A2.

The oct-2 gene contains a functional homeo box domain.

The two oct-2 partial cDNAs were sequenced and found to be identical in their overlap. The predicted oct-2 protein sequence was compared to all possible translations of the GenBank Genetic Sequence Data Bank and similarities were seen with proteins containing the so-called homeo box domain. The homeo box is a 60 amino acid domain which was originally found in many developmentally important Drosophila proteins and in yeast mating type proteins that control gene expression in a cell type-specific fashion. These homeo box proteins have been shown to be sequence-specific DNA binding proteins which act as "master switches" by controlling gene expression and thus cellular differentiation. Human and mouse genes have been identified which contain homeo boxes but the function of these genes is unknown. The oct-2 sequence showed that it is evolutionarily divergent from the previously cloned human homeo box genes and futhermore, it is located on a different human chromosome (#19). Site directed mutation of the homeo box domain destroyed binding to the octamer motif, thus demonstrating for the first time that a human homeo box protein is a sequence-specific DNA binding protein. We noted that the binding sites of the yeast and Drosophila homeo box proteins were similar to the Ig octamer motif and showed that the oct-2 protein also bound to these sequences. Thus, all homeo box proteins may have a binding preference for A-T rich, Ig octamer-like sequences. In summary, it is tempting

to speculate that the oct-2 gene product, on the basis of its homeo box homology, may play an important role in development in the lymphoid system.

Publications

Wirth T, Staudt LM, Baltimore D: An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity. Nature 1987;329:174.

Staudt LM, Clerc RG, Singh H, Lebowitz JH, Sharp PA, Baltimore D. Molecular cloning of a lymphoid-specific cDNA encoding a protein that binds to the regulatory octamer DNA motif. Science 1988, in press.

Ko HS, Fast P, McBride W, Staudt LM. A human protein specific for the immunoglobulin octamer DNA motif contains a functional homeo box domain. Cell 1988, submitted.

SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY BRANCH
DCBD, NCI

October 1, 1987 through September 30, 1988

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:

We have continued our studies of the immunological functions of cells of the epidermis. We have continued to utilize short-term cultured epidermal Langerhans cells in a primary in vitro sensitization system. We are thereby able to circumvent complex in vivo interactions in order to generate specific T cell responses from virginal T cells. After generating such primary responses to highly reactive chemicals, we have recently been successful at generating protein specific responses in such virginal T cells. We are currently attempting to generate viral specific responses in vitro. The cytokine secreting characteristics of the T cell lines which have been generated are being characterized. Most of them are IL-4 producing Th_2 cells.

For several years, we have also been studying the immunological function of class II bearing keratinocytes. Although we have demonstrated that they can elicit secondary responses in alloreactive T cells and can present immunogenic peptides to T cell hybridomas, they perform these functions poorly. Recently, we have demonstrated that these class II bearing keratinocytes can induce specific immunological unresponsiveness in hapten-specific T cell clones.

Molecular Basis of Autoimmune Skin Diseases:

This laboratory studies 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. Specifically, antibodies in these diseases define molecules in the normal epidermis. We have characterized the antigens defined by antibodies in the sera of patients with three of these diseases: bullous pemphigoid, pemphigus vulgaris (PV) and pemphigus folaceus (PF). We have demonstrated that PF antibodies bind a protein found in desmosomes, and define a calcium-sensitive epitope on a desmosomal protein complex. Thus, PF is an autoimmune disease in which the antibody target is the desmosome. We have demonstrated that antibodies from

all PF patients bind a unique complex of proteins that is distinct from the complex bound by PV antibodies.

In order to better define some of these epidermal antigens, we have developed a lambda gt-11 expression library, from which we have isolated a cDNA clone that synthesizes part of the bullous pemphigoid (BP) antigen. We have used this BP clone to show that the mRNA encoding BP antigen is about 9kb. We We have also deduced the peptide sequence of the C terminal end of the molecule.

Analysis of Chemical Mediators of Inflammation:

We have focused a major effort on the study of the human dermal microvascular endothelial cell, its cultivation, characterization and the modulation of its phenotypic and functional characteristics. Human umbilical vein endothelial cells and human dermal microvascular endothelial cells can be isolated and grown in pure cultures.

We have induced human dermal microvascular endothelial cells (HDMEC) to undergo extremely rapid (< 1 hr) differentiation. The HDMEC form elongated tubes that contain lumens. This angiogenesis is induced by matrigel, a basement membrane-like, biochemically complex gel extracted from EHS sarcoma. We have defined the substance in matrigel that constitutes the major angiogenic signal. This substance is laminin. In reconstitution experiments, we have found that collagen I gels supplemented with purified laminin, but not collagen IV, support significant endothelial cell differentiation. Blockade experiments using laminin antibodies or the synthetic peptide YIGSR inhibit endothelial cell differentiation on matrigel.

We have defined the immunologic phenotype of HDMEC. These cells possess the cell adhesion molecules ICAM-1, LFA-3 and CD44. ICAM-1 can be upregulated in a time and dose response fashion by IL-1 and TNF but LFA-3 and CD44 are not. In addition, these cells also express CD13, CD9, CD6 and CDw26 which were previously thought to be present only on T cells. We have demonstrated that adhesion of T cells to HDMEC is due to expression of cell adhesion molecules on both types of cells and that it can be selectively inhibited by specific monoclonal antibodies directed against cell adhesion molecules. Key cell adhesion molecules in this regard are CD2 and LFA1 on T cells and ICAM1 and CD44 on HDMEC.

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. Two patients with a history of multiple basal cell carcinomas were treated with oral isotretinoin for 7-8 years with inhibition of new tumor formation. Treatment was stopped in order to determine the need for chronic therapy: one patient with the nevoid basal cell carcinoma syndrome developed more than 29 tumors within 13 months and the second, with arsenic-induced tumors, developed one new tumor as of the 30-month visit. The efficacy of isotretinoin as a chemopreventive agent is being studied further in a series of seven patients with xeroderma pigmentosum

and nevoid basal cell carcinoma syndrome. Results indicate significant partial inhibiton of new tumor formation during therapy and a marked increase in new tumor formation after therapy. Long-term therapy with low-dose isotretinoin is now being used for cancer chemoprevention in these patients. The need for continuous maintenance therapy for chemoprevention of skin cancer with isotretinoin may vary with the underlying etiology (genetic or environmental) of the patient's tumors.

Studies of DNA Repair in Normal Human Cells from Patients with Xeroderma Pigmentosum and Neurodegenerative Disorders:

We are continuing our studies of the role of DNA repair processes in cancer, aging and neurodegenerative disorders. In collaboration with Dr. Kenneth H. Kraemer, we are able to successfully transfect human lymphoblastoid cell lines with a plasmid (pRSVcat) which contains the bacterial cat gene coding for chloramphenicol acetyltransferase (CAT). The plasmid is irradiated with different doses of 254-nm UV radiation. Then, in order to evaluate the repair of dimer and nondimer photoproducts, aliquots containing the irradiated plasmid are treated with photoreactivating enzyme (E. Coli photolyase) and light, a treatment which monomerizes the UV-induced cyclobutane-type of pyrimidine dimer and leaves only nondimer photoproducts. We have preliminary data on a normal cell line with high post-UV-radiation survival. Of the 3 cell lines, the normal one showed the greatest CAT activity after its transfection with the irradiated plasmid. The Cockayne syndrome (CS) cell line transfected with the irradiated plasmid had a low level of CAT activity which was not increased when the irradiated plasmid was photoreactivated prior to transfection. The XP cell line transfected with the irradiated plasmid has the lowest CAT activity. This activity was increased, although never to normal levels, after the plasmid had been photoreactivated prior to transfection. Our results suggest that the CS cells repair all the dimers normally but do not repair the nondimer photoproducts. Our results also suggest that the XP cells do not repair all the dimers and do not repair all the nondimer photoproducts. We plan to continue these studies with cell lines from additional patients and to adapt the assay for measuring the effects of the ionizing-radiation-type of DNA damage which would make the assay suitable for testing the DNA-repair capacity of radiosensitive neurodegenerations such as Alzheimer's disease and Parkinson's disease.
With Dr. Miral Dizdaroglu and Dr. Ewa Gajewski, we are using capillary gas chromatography-mass spectrometry to detect various types of damage induced by ionizing radiation in DNA. We have completed a study of the effects of DNA conformation on the formation of 8,5'-cyclo-purine 2'-deoxyribonucleoside residues in isolated DNA irradiated in vitro. We are now studying the possibility of repair of these lesions in irradiated living cells.

Chemistry, Structure, and Biosynthesis of Mammalian Epidermal Keratin Filaments:

We are continuing our studies on the biosynthesis, structure and functions of epidermal cytoskelatal proteins, the principal products of human and mouse epidermis. The availability of complete amino acid sequences of several keratin chains has let us to propose more advanced models of intermediate filament structure. Even though filaments are polymorphic, a major structural form consists of 8 4-chain molecules, arranged in an antiparallel mode. The

structure of the 4-chain molecule is being examined by scanning transmission electron microscopy (with Dr. Hainfeld) using platinum-clathrates complexed onto cystine residues. In collaboration with Drs. A.C. Steven, David Parry and R.D.B. Fraser, we have constructed "surface-lattice" models of filament structure in which the rod domains of the protein chains form the basic filament core, and the variable end domains occupy peripheral positions. Limited proteolytic cleavage experiments have provided direct evidence in support of these structural models and of the precise alignment of the neighboring rod domains. In addition, scanning transmission electron microscopy is being used to explore the mass, structure and association of 4-chain units in an attempt to describe intermediate levels of filament substructure. However, the structural organization of the end domains is still uncertain. In the case of the keratins, we propose that their glycine-serine-rich sequences form omega-loop structures and interact with other cellular components including filaggrin and the cell envelop by H-bonding and disulfide cross-links. In collaboration with Dr. James Mack, these ideas are being tested by use of solid-state NMR of keratin filaments isotopically labeled with glycine (to study end domains) and leucine (to study the rod domain). Preliminary data suggests the glycine-rich domains have little or no structural order, which however, we expect should change when filaments are complexed with filaggrin.

Dr. Rothnagel has now isolated a major portion of the mouse filaggrin gene from a cosmid library, which contains 17 repeating units interspersed by one or two apparently randomly distributed tyrosine-rich linker sequences. He will construct a new cosmid library from genomic DNA restricted to completion with Dra I in an attempt to isolate the full-length gene including upstream regulatory sequences. A cDNA isolate clone for human filaggrin has now been isolated from lambda gtll expression library. This has been completely sequenced and demonstrated the presence of a 972 bp repeat different from mouse filaggrin. The full-length gene will also be isolated from a genomic library and human cosmid library. Dr. McKinley-Grant is using in situ hybridization techniques to study the expression of the human filaggrin in normal and abnormally keratiniziang epidermis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03657-14 D

PERIOD COVERED

October 1, 1987 to September 30, 1988

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)*

P.I.: S.I. Katz, Branch Chief, Dermatology Branch, DCBD, NCI

OTHER: W. Caughman, Senior Staff Fellow, Dermatology Branch, DCBD, NCI
A. Gaspari, Medical Staff Fellow, Dermatology Branch, DCBD, NCI
T. Furue, Visiting Fellow, Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(if any)*

Dermatology Branch, USUHS, Bethesda
Immunology Branch, DCBD, NCI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7	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.)*

The major area of study of this laboratory is the role of the epidermis as an immunological organ. We have found that epidermal Langerhans cells are derived from precursor cells in the bone marrow and play an essential role in many of the immunological reactions affecting the skin. We have also identified an epidermal Interleukin 1-like cytokine which may serve as a second signal in the generation of T cell responses as well as a proinflammatory agent affecting other cells - especially neutrophils. We have demonstrated that when epidermal Langerhans cells are cultured for 2-3 days they become very potent antigen presenting cells compared to freshly prepared Langerhans cells. We have therefore utilized cultured Langerhans cells for the generation of primary immune responses in resting unsensitized T cells. We have demonstrated that when cultured cells are modified with hapten, they can generate primary immune responses. The sensitized T cells thus generated respond preferentially to the same hapten in vitro We have also concentrated our efforts on the characterization of murine dendritic thy 1 positive epidermal cells. We have utilized highly enriched populations of freshly prepared cells and identified their T cell nature by demonstrating that they express γδ-like T cell receptor along with the associated T3 components. As these cells are also present in nude mice they may represent an extra thymic source of T cells in nude as well as normal mice. The other major focus of this laboratory has been the study of the function of class II MHC bearing keratinocytes which appear in humans and mice during cell-mediated reactions in the skin. We have demonstrated that these cells can 1) present peptide fragments to T cell hybridomas, 2) serve as targets for class II specific cytotoxic T lymphocytes, and 3) induce secondary alloreactive T cell responses, although the first and third are performed only poorly. Our recent studies demonstrate that these class II bearing keratinocytes induce specific immunological unresponsiveness.

GPO 914-916

Project Description

Major Findings:

We have found that when epidermal Langerhans cells are cultured for 2-3 days they become very potent antigen presenting cells for hapten modified self as well as for protein antigens. When cultured, these cells express 10-18 times as much Class II antigen as do freshly prepared cells. Although we had previously failed to induce primary immune responses utilizing freshly prepared Langerhans cells in vitro, we repeated these studies using cultured Langerhans cells which had been modified by hapten. After depleting the responder T cell population of autologously reacting cells we were able to generate primary proliferative as well as IL-2 responses by the previously resting, nonsensitized T cells. Utilizing these cells in secondary immune responses in vitro, we found that the T cells responded preferentially to the hapten to which they were "primed". We have produced T cell lines from these in vitro sensitized cells and virtually all of them produce IL-4 as opposed to IL-2. We are currently attempting to enhance the growth of IL-2 producing T cells since these may be more useful in vivo. These studies will hopefully result in our ability to generate primary in vitro responses to protein and tumor associated antigens.

We have continued our studies of the murine thy 1^+ dendritic epidermal cells which are bone marrow derived. Using freshly isolated cells in C3H mice we found that they expressed the protein products of the $\gamma\delta$-like T cell receptor heterodimers and the associated T3 components. These findings conclusively characterize these cells of being of T cell lineage despite their not expressing mature T cell markers. We have cultured the thy 1^+ dendritic cells from the skin of nude mice and have found that they express the entire T cell antigen receptor on their membrane. The T3 complex differs from that seen on mature T cells. The cells may therefore serve as an extra thymic source of T cells in nude as well as normal mice.

Other major interests and studies include 1) those characterizing the functions of class II-MHC bearing keratinocytes, and 2) the assessment of the effect of cyclosporine A on the antigen-presenting and accessory cell functions of Langerhans cells. With regard to the former, we have developed techniques which can deplete Langerhans cells from class II bearing keratinocytes so that the function of these keratinocytes, which are induced by injecting interferon gamma, can be assessed. We found that they function 1) as targets for class II specific cytotoxic T cells, 2) as stimulators in secondary allogeneic T cell proliferative responses, and 3) in the presentation of small peptide antigens to T cell hybridomas. Our most recent studies have demonstrated that these class II bearing keratinocytes when hapten-coupled, induce specific immunological unresponsiveness in hapten-specific T cell clones.

Studies assessing the effects of cyclosporine A (CSA) on antigen presenting and accessory cell functions of Langerhans cells have demonstrated that CSA interacts with Langerhans cells after a 2 hour incubation and renders them (Langerhans cells) relatively unable to perform many of the T cell stimulatory

functions. In addition, CSA has a profound effect on the proliferation of epidermal cells. We have also studied the effects of dexamethasone on epidermal Langerhans cells. From these in vitro studies, it appears that dexamethasone is cytolytic to a subpopulation of Langerhans cells.

Publications:

Breathnach SM, Katz SI. Immunopathology of cutaneous graft-versus-host disease, Am J Dermatopathology 1987;9:343-348.

Caughman SW, Krieg T, Timpl R, Hintner H, Katz SI. Nidogen and heparan sulfate proteoglycan: Detection of newly isolated basement membrane components in normal and epidermolysis bullosa skin, J Invest Dermatol 1987;89:547-550.

Gaspari AA, Lotze MT, Rosenberg SA, Stern JB, Katz, SI. Dermatologic changes associated with interleukin-2 administration, JAMA 1987;258:1624-1629.

Shimada S, Caughman SW, Sharrow SO, Stephany D, Katz SI. Enhanced antigen presenting capacity of cultured Langerhans cells is associated with markedly enhanced expression of Ia antigen, J Immunol 1987;139:2551-2555.

Shimada S, Katz, SI. The skin as an immunological organ, Arch of Path & Lab Med 1988;112:231-234.

Sakamoto K, Sachs DH, Shimada S, Popitz-Bergez, FA, Pennington, LR, Pescovitz, MD, McDonough MA, MacVittie TJ, Katz SI, Gress RE. Bone marrow transplantation in miniature swine, Transplantation 1988 (in press).

Furue M, Katz SI. Cyclosporin A inhibits accessory cell and antigen-presenting cell functions of epidermal Langerhans cells, Transplantation Proceedings 1988; :87-91.

Furue M, Katz SI. Moleucles on activated human T cells, Am J Dermatopath (in press).

Furue M, Gaspari AA, Katz SI. The effect of cyclosporine A on epidermal cells II. Cyclosporine A inhibits proliferation of normal and transformed keratinocytes, J Invest Dermatol 1988;90:796-800.

Gaspari AA, Katz SI. Induction and functional characterization of class II major histocompatibility complex (Ia) antigens on murine keratinocytes, J Immunol 1988;140:2956-2963.

Hauser C, Katz SI. Activation and expansion of hapten-and protein specific T helper cells from nonsensitized mice, Proc Nat Acad Science (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03667-04 D

PERIOD COVERED

October 1, 1987 to September 30, 1988

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)*

P.I.: John R. Stanley, M.D., Dermatology Branch, DCBD, NCI

OTHER: Stephan Muller, M.D., Visiting Fellow, Dermatology Branch, DCBD, NCI
Neil Korman, M.D., Medical Staff Fellow, Dermatology Branch, DCBD, NCI
Toshihiro Tanaka, M.D., Medical Officer, Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	4	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 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. 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 BP, EBA, PV or PF in various complicated cases of these diseases. We have demonstrated that PF autoantibodies can cross the placenta and bind to fetal skin without causing disease in the newborn. We have demonstrated that PF antibodies bind a protein (desmoglein I) found in desmosomes, and define a calcium-sensitive epitope on a desmosomal protein complex. Thus, PF is an autoimmune disease in which the antibody target is the desmosome. We have demonstrated that antibodies from all PF patients bind a unique complex of proteins that is distinct from the complex bound by PV antibodies. Although the PV antigen complex is distinct from the PF antigen complex, the two complexes share certain biochemical similarities. For example, both complexes have a common 85 D polypeptide chain which binds to a 160 kD (PF) or 130 kD (PV) polypeptide. The latter two polypeptides have identical isoelectric points. Using a lambda gt11 expression library, we have isolated a cDNA clone that synthesizes part of the BP antigen. We have used this BP clone to show that the mRNA encoding BP antigen is about 9 kb. We have also deduced the peptide sequence of the C-terminal end of the molecule.

PHS 6040 (Rev. 1/84)

GPO 814-816

Project Description

Major Findings:

Antibodies from all pemphgus foliaceus (PF) patients define a complex of polypeptides that includes desmoglein I, a known core desmosomal glycoprotein. PF is an autoimmune disease in which the desmosome appears to be the target of the autoantibody. Antibodies from PF patients define a calcium-sensitive conformational epitope on this desmosomal protein complex.

Certain epitopes of the desmosomal glycoprotein desmoglein I are expressed only in stratified squamous epithelia.

Pemphigus vulgaris (PV) autoantibodies bind a complex of polypeptides that is different from the PF antigen complex, so that these diseases can be diagnosed and differentiated at a molecular level.

Although the PV and PF antigen complexes are distinct, they share certain biochemical similarities. In the PF antigen complex an 85 kD polypeptide is disulfide and noncovalently bound to a 160 kD polypeptide (desmoglein I) to form a 260 kD molecule. Similarly, in the PV antigen complex, the same 85 kD polypeptide is bound to a 130 kD polypeptide to form a 210 kD molecule. The PF 160 kD and the PV 130 kD polypeptide have the same isoelectric point.

A cDNA clone with coding sequences for the C-terminal end of BP antigen has been isolated by screening a cDNA lambda gt11 library with BP autoantibodies.

The mRNA encloding BP antigen is 9kb.

Human dermal fibroblasts synthesize laminin.

Publications:

Rubinstein N, Stanley JR. Pemphigus foliaceus antibodies and a monoclonal antibody to desmoglein I demonstrate stratified squamous epithelial-specific epitopes of desmosomes, Am J Dermatopath 1987;9:510-514.

Eyre RW, Stanley JR. Human autoantibodies against a desmosomal protein complex with a calcium-sensitive epitope are characteristic of pemphigus foliaceus patients, J Exp Med 1987;165:1719-1724.

Eyre RW, Stanley JR. Maternal pemphigus foliaceus with cell surface antibody bound in neonatal epidermis, Arch Dermatol 1988;124:25-27.

Eyre RW, Stanley JR. Identification of pemphigus vulgaris antigen extracted from normal human epidermis and comparison with pemphigus foliaceus antigen, J Clin Invest 1988;81:807-812.

Woodley DT, Stanley JR, Reese MJ, O'Keefe EJ. Human dermal fibroblasts synthesize laminin, J Invest Dermatol 1988;90:679-683.

Mueller SM, Stanley JR. Similarities and differences between the two major types of pemghigus: pemphigus vulgaris and pemphigus foliaceus. In: Wojneroswshi F, Briggaman RA, eds. The Management of Blistering Diseases. London: Chapman and Hall, (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03666-10 D

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters 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, laboratory, and institute affiliation)*

PI: Thomas J. Lawley, M.D., Dermatology Branch, DCBD, NCI

OTHER: Robert Swerlick, M.D., Medical Staff Fellow, Dermatology Branch, DCBD, NCI
Carol McNeely, M.D., Medical Staff Fellow, Dermatology Branch, DCBD, NCI
Edith Garcia-Gonzalez, Visiting Fellow, Dermatology Branch, DCBD, NCI
Joseph Cason, Technician, Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(if any)*

LCI, NIAID

LAB/BRANCH

Dermatology Branch

SECTION

NCI, NIH, Bethesda, Maryland 20892

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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 space provided.)*

The precise role of endothelial cells in inflammation is unknown. However, it is likely that they are important in initiating, sustaining and terminating inflammation by secretion of proinflammatory substances, modulation of immunologically relevant cell surface antigens and receptors and regulation of leukocyte adherence and diapedesis. We have developed techniques to isolate and grow pure cultures of human dermal microvascular endothelial cells (HDMEC) and are conducting an in depth anlaysis of their role in cutaneous inflammation. We also have developed techniques to measure in parallel biologic function (cell adherence) and cell surface expression of immunologically relevant molecules by HDMEC. We have found that these cells express specific cell adhesion molecules on their surface and that these molecules play a critical role in leukocyte endothelial cell adherence reactions. Moreover, we find the expression of these molecules can be upregulated by exposure of the HDMEC's to biologic response modifiers such as IL-1, TNF and IFN-γ . This upregulation is both time and dose responsive and is specific for selected cell adhesion molecules. The substances that stimulate HDMEC are also selective in that they do not upregulate purified human T-cells. Conversely HDMEC are not effected by T cell stimulatory molecules. We have also succeeded in causing HDMEC to undergo rapid differentiation into capillary-like structures by culturing on a matrix known as matrigel. We have defined the key molecule in this complex matrix which acts as the signal for HDMEC angiogenesis. We are investigating the influence of differentiation on the immunological function of HDMEC.

Project Description

Major Findings:

1) We have induced human dermal microvascular endothelial cells (HDMEC) to undergo extremely rapid (< 1 hr) differentiation. The HDMEC form elongated tubes that contain lumens. This angiogenesis is induced by matrigel, a basement membrane-like, biochemically complex gel extracted from EHS sarcoma.

2) We have defined the substance in matrigel that constitutes the major angiogenic signal. This substance is laminin. In reconstitution experiments, we have found that collagen I gels supplemented with purified laminin, but not collagen IV, support significant endothelial cell differentiation. Blockade experiments using laminin antibodies or the synthetic peptide YIGSR inhibit endothelial cell differentiation on matrigel.

3) We have defined the immunologic phenotype of HDMEC. These cells possess the cell adhesion molecules ICAM-1, LFA-3 and CD44. ICAM-1 can be upregulated in a time and dose response fashion by IL-1 and TNF but LFA-3 and CD44 are not. In addition, these cells also express CD13, CD9, CD6 and CDw26 which have previously thought to be present only on T cells.

4) We have demonstrated that adhesion of T cells to HDMEC is due to expression of cell adhesion molecules on both types of cells and that it can be selectively inhibited by specific monolconal antibodies directed against cell adhesion molecules. Key cell adhesion molecules in this regard are CD2 and LFA1 on T cells and ICAM1 and CD44 on HDMEC.

5) We have defined the proinflammatory capabilities of purified human C5a des Arg in human skin. It is 5 to 50x less potent than native C5a in causing cutaneous inflammation.

6) We have selectively depleted mast cells from human skin and using it as a substrate, dissected the anaphylatoxic and chemotactic properties of C5a *in vivo*.

7) We have demonstrated that C5a induced immediate hypersensitivity reactions are selectively dependent on normal numbers of circulating polymorphonuclear neutrophils in studies of patients of bone marrow failure.

8) C5a induces the secretion of IL-1 by human monocytes, is more potent than C5a des Arg and acts synegistically in this regard with IFN-γ or endotoxin.

Publications:

Hinter H, Romani N, Stanzl U, Fritsch, Lawley TJ. Phagocytosis of keratin filament aggregates following opsonization with IgG-anti-keratin filament autoantibodies, J Invest Dermatol 1987;88:176-182.

Kubota Y, Gaither TA, O'Shea JJ, Lawley TJ. Herpes simplex virus type 1 infection induces the formation of Fc IgG and C3b receptors on keratinocytes and A431 cells and endothelial cells, J Immunol 1987;138:1137-1142.

Yancey KB, Bielory L, Wright R, Young N, Frank MM, Lawley TJ. Patients with bone marrow failure demonstrate decreased cutaneous reactivity to human C5a, J Invest Dermatol 1987;88:388-392.

Yancey KB, Swerlick RA, Lawley TJ. C5a as a mediator of cutaneous inflammation, Am J Dermatopathol 1987;9:138-143.

Koski CL, Sanders ME, Swoveland PT, Lawley TJ, Shen ML, Frank MM, Joiner KA. Activation of terminal components of complement in patients with Guillain-Barre Syndrome and other demyelinating neuropathies, J Clin Invest 1987;80:1492-1497.

Okusawa S, Dinarello CA, Endres S, Yancey KB, Lawley TJ, Frank MM, Burke JF, Gelfand JA. C5a induction of human IL-1: Synergistic effect with endotoxin or interferon-gamma, J Immunol 1987;139:2635-2640.

Zeitz M, Hopt U, Wust B, Galanos C, Moller B, Lawley TJ, Riecken EO. Absence of complement fixing antibodies against lipopolysaccharides from Escherichia coli in a subgroup of patients with Crohn's disease, Gut 1987;28:1460-1466.

Keahey TM, Yancey KB, Lawley TJ. Immediate hypersensitivity, J Am Acad Dermatol 1987;17:826-837.

Yarchoan R, Perno CF, Thomas RV, Klecker RW, Allain JP, Wills RJ, McAtee N, Fischl MA, Dubinsky R, McNeeley MC, Mitsuya H, Pluda JM, Lawley TJ, Leuther M, Safai B, Collins JM, Myers CE, Broder S. Phase I Studies of 2', 3' -Dideoxycytidine in severe human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT), Lancet 1988;1:76-80.

Bielory L, Gascon P, Lawley TJ, Young NS, Frank MM. Human serum sickness: a prospective analysis of 35 patients treated with equine anti-thymocyte globulin for bone marrow failure, Medicine 1988;67:40-57.

Swerlick RA, Yancey KB, Lawley TJ. A direct in vivo comparison of the inflammatory properties of human C5a and C5a des Arg in human skin, J Immunol 1988;140:2376-2381.

Yancey KB, Lawley TJ, Harvath L. An analysis of the interaction of human C5a and C5a des Arg with human monocytes and neutrophils: Flow cytometric and cytometric and chemotaxis studies, J Invest Dermatol (in press).

Kubota Y, Kleinman H, Martin GR, Lawley TJ. The role of laminin and basement membrane in the differentiation of human endothelial cells into capillary-like structures, J Cell Biol (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03659-14 D

PERIOD COVERED

October 1, 1987 to September 30, 1988

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)*

P.I.: G.L. Peck, Senior Investigator, Dermatology Branch, DCBD, NCI

OTHER: J.J.DiGiovanna, Senior Staff Fellow, Dermatology Branch, DCBD, NCI
E. Toombs, Medical Staff Fellow, Dermatology Branch, DCBD, NCI
I. Tokar, Registered Nurse, Dermatology Branch, DCBD, NCI
K. Kraemer, Senior Investigator, Cell Genetics Br., NCI

COOPERATING UNITS *(if any)*

Cancer Prevention Studies Branch, DCPC, NCI, NIH, Bethesda, Maryland 20892

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.9	3.9	

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. Psychological testing revealed decreased anxiety and depression in cystic acne patients after treatment with isotretinoin. One chronic toxicity, "retinoid hyperostosis," characterized by anterior spinal ligament calcification and osteophyte formation of vertebrae, and extraspinal tendon and ligament calcification has been observed in 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. Extraspinal tendon and ligament calcification also occurred commonly after chronic therapy for psoriasis and disorders of keratinization with etretinate, an aromatic retinoid. The usual sites of involvement were the ankles, pelvis and knees. Due to storage in fat, serum levels extretinate can be measured for prolonged periods (> 2 years) after discontinuation of therapy.

Seven patients with xeroderma pigmentosum were entered into a cancer chemoprevention study using isotretinoin: results indicate significant partial inhibition of new tumor formation during therapy and a marked increase in new tumor formation after therapy. Long-term therapy with low-dose isotretinoin is now being used for cancer chemoprevention in these patients. The need for continuous maintenance therapy for chemoprevention of skin cancer with isotretinoin may vary with the underlying etiology (genetic or environmental) of the patient's tumors.

GPO 914-918

Project Description

Major Findings:

Two patients with a history of multiple basal cell carcinomas were treated with oral isotretinoin for 7-8 years with inhibition of new tumor formation. After treatment was stopped in order to determine the need for chronic therapy, one patient with the nevoid basal cell carcinoma syndrome developed 29 tumors within 13 months. The second patient with arsenic-induced tumors developed one new tumor as of the 30-month visit. This indicates that the need for continuous maintenance therapy for chemoprevention of skin cancer with isotretinoin may vary with the underlying etiology (genetic or environmental) of the patients tumors. The efficacy of isotretinoin at high dose as a chemopreventive agent is being studied further in a series of 7 patients with xeroderma pigmentosum and nevoid basal cell carcinoma syndrome. Preliminary results indicate significant partial, but not complete, efficacy in inhibiting new tumor formation. Furthermore, impressive increases in the rate of new tumor formation have been observed after stopping therapy. Low-dose isotretinoin is now being used in these patients in order to minimize toxicity.

Retinoid hyperostosis, a variant form of diffuse idiopathic skeletal hyperostosis, was observed in patients with multiple basal cell carcinomas treated with high doses (1.5 mg/kg/day or higher) of isotretinoin for a minimum of 2 years. Severity of this disorder, defined by anterior spinal ligament calcification and osteophyte formation at 2 or more vertebral levels without disc space narrowing, appeared to be dose-dependent. Isotretinoin induced calcification of both spinal ligaments and extraspinal ligaments and tendons. Extraspinal tendon and ligament calcification also occurred commonly after chronic therapy for psoriasis and disorders of keratinization with etretinate, an aromatic retinoid. The usual sites of involvement were the ankles, pelvis and knees.

Detectable serum concentrations of etretinate were observed for more than two years following discontinuation of therapy. The median half-life of etretinate in the serum was calculated to be 12.5 weeks - overweight patients tended to have shown elimination, maintain higher serum concentrations and clear etretinate later.

While no excess psychiatric morbidity was observed in a group of patients with cystic acne, substantial evidence of psychologic distress was noted prior to treatment. Significant reductions in anxiety were observed following treatment, with mitigation of anxiety and depression most robust in those patients with the greatest dermatologic improvement on isotretinoin. Clinical interviews after treatment revealed lessened preoccupation with details of hygiene and concern about appearance and improvement in feelings of embarrassment and selfconsciousness.

One patient with nevus comedonicus covering most of the left side of his trunk had an inadequate response to isotretinoin but responded excellently to topical ammonium lactate lotion.

Publications:

Rubinow DR, Peck GL, Squillace KM, Gantt PG. Reduced anxiety and depression in cystic acne patients after successful treatment with oral isotretinoin, J Am Acad Dermatol 1987;17:25-32.

Peck GL. Long-term retinoid therapy is needed for maintenance of cancer chemopreventive effect, Dermatologica 1987;175(suppl 1):138-144.

DiGiovanna JJ, Peck GL. Retinoid toxicity, Progress in Dermatology 1987;21 (suppl 3):1-8.

DiGiovanna JJ, Peck GL. Pityriasis rubra pilaris. In: Provost TT, Farmer ER, eds. Current Therapy in Dermatology. Burlington, Ontario: Becker, 1987;6-10.

Peck GL, Vahlquist A, Gollnick H. Retinoids - present status, in Dermatology in Five Continents. In: Organos CE, Stadler R, Gollnick H, eds. Proceedings of the XVII World Congress of Dermatology, Berlin. Berlin: Springer-Verlag, 1988;491-494.

Sarnoff DS, Peck GL. Therapy of basal cell carcinoma. In: Magrath IT, ed. New Directions in Cancer Treatment. Heidelberg: Springer-Verlag, (in press).

Milton GP, DiGiovanna JJ, Peck GL. Treatment of nevus comedonicus with ammonium lactate lotion, J Am Acad Dermatol (in press).

Kraemer KH, DiGiovanna JJ, Moshell AN, Tarone RE, Peck GL. Prevention of skin cancer with oral 13-cis-retinoic acid in xeroderma pigmentosum, New Eng J Med (in press).

Peck GL, DiGiovanna JJ, Sarnoff DS, Gross EG, Butkus D, Olsen T, Yoder F. Treatment and prevention of basal cell carcinoma with isotretinoin, J Am Acad Dermatol (in press).

DiGiovanna JJ, Zech LA, Ruddel ME, Gantt G, Peck GL. Etretinate: persistent serum concentrations after long-term therapy, Arch Dermatol (in press).

DiGiovanna JJ, Helfgott R, Gerber LH, Peck GL. Extraspinal tendon and ligament calcification after long-term isotretinoin therapy, (submitted for publication).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03630-18 D

PERIOD COVERED

October 1, 1987 to September 30, 1988

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)*

P.I.: G.L.Peck, Senior Investigator, Dermatology Branch, DCBD, NCI

OTHER: J.J. DiGiovanna, Senior Staff Fellow, Dermatology Branch, DCBD, NCI
E. Toombs, Medical Staff Fellow, Dermatology Branch, DCBD, NCI
T. Mehrel, Visiting Fellow, Laboratory for Cellular Carinogenesis and Tumor Promotion, DCE, NCI

COOPERATING UNITS *(if any)*

Laboratory for Cellular Carcinogenesis and Tumor Promotion, DCE, NCI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	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.)*

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 and malignant 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 normal human skin, newborn mouse 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 limb skin.

Using molecular hybridization probes specific for transcripts of individual keratin genes, keratin gene expression in skin cancer and cutaneous disorders of keratinization indicate the following. In contrast to normal epidermis where the expression of proliferation-associated keratin genes is suppressed after cells migrate from the basement membrane, hyperproliferative disorders of the epidermis exhibit inappropriate expression of proliferation-associated keratin genes in the more superficial layers of the epidermis. In addition, skin cancers fail to express differentiation-associated keratins, indicative of their undifferentiated state.

Project Description

Major Findings:

A project studying keratin gene expression in skin cancer and cutaneous disorders of keratinization has been initiated. The study employes molecular hybridization probes that are specific for transcripts of individual keratin genes. The transcripts are localized at the cellular level within frozen sections by in situ hybridization with 35-S labeled RNA probes. Diseases studied to date include Darier's disease, psoriasis, lamellar ichthyosis, basal cell carcinoma and squamous cell carcinoma. Preliminary findings suggest that, in contrast to normal epidermis where the expression of proliferation-associated keratin genes is suppressed after cells migrate from the basement membrane, hyperproliferative disorders of the epidermis exhibit inappropriate expression of proliferation-associated keratin genes in the more superficial layers of the epidermis. In addition, skin cancers fail to express differentiation-associated keratins, indicative of their undifferentiated state. Recent experiments on normal skin indicate a sequential expression of differentiation-associated keratins (K1, K10).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 CB 03638-19 D

PERIOD COVERED

October 1, 1987 to September 30, 1988

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)*

PI: J. H. Robbins, M.D., Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(if any)*

Laboratory of Molecular Carcinogenesis, DCE, NCI
Radiation Oncology Branch, DCT, NCI
Biostatistics Branch, DCCP, NCI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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.)*

Studies in this laboratory are designed to elucidate the role of DNA repair processes in carcinogenesis and in normal and abnormal aging and in neurodegeneration. 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 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, Friedreich ataxia, and Cockayne syndrome (CS). These studies are designed to elucidate the pathogenesis of these disorders. We assess the biological effectiveness of DNA repair by 1) in vitro assays of cell survival after treatment of the cells with DNA-damaging agents; 2) analysis of chromosome and chromatid aberrations in cells treated with DNA-damaging agents; and 3) transfection studies using irradiated plasmids. We search for DNA damage by 1) extracting the DNA and having it subjected to analysis by capillary gas chromatography-mass spectrometry; and 2) studying unscheduled DNA synthesis induced in cultured cells by chemical carcinogens. We search in patient cells for abnormalities in DNA-repair genes and in their DNA-damage inducible transcripts using cDNA probes in Southern and Northern blot hybridization studies.

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

Project Description

Other Professional Personnel:

M.L. Dirksen, Ph.D., Expert, Dermatology Branch, DCBD, NCI
M. Dizdaroglu, Ph.D., Research Chemist, NBS
E. Gajewski, Ph.D., Research Chemist, NBS
A.J. Fornace, Jr., M.D., Senior Investigator, RO, NCI
K.H. Kraemer, M.D., Senior Investigator, CH, NCI
J. Nove, Research Specialist, Laboratory of Radiobiology, Harvard School of Public Health
L. Seguin, Ph.D., Consultant, Dermatology Branch, DCBD, NCI
R.E. Tarone, Methematical Statistician, B, NCI

Major Findings:

In collaboration with Dr. Kenneth H. Kraemer, we are able to successfully transfect human lymphoblastoid cell lines with a plasmid (pRSVcat) which contains the bacterial cat gene coding for chloramphenicol acetyltransferase (CAT). The plasmid is irradiated with different doses of 254-nm UV radiation. Then, in order to evaluate the repair of dimer and nondimer photoproducts, aliquots containing the irradiated plasmid are treated with photoreactivating enzyme (E. coli photolyase) and light, a treatment which monomerizes the UV-induced cyclobutane-type of pyrimidine dimer and leaves only nondimer photoproducts. We have preliminary data on a normal cell line with high post-UV-radiation survival. Of the 3 cell lines, the normal one showed the greatest CAT activity after its transfection with irradiated plasmid. The CS cell line transfected with the irradiated plasmid had a low level of CAT activity which was not increased when the irradiated plasmid was photoreactivated prior to transfection. The XP cell line transfected with the irradiated plasmid had the lowest CAT activity. This activity was increased, although never to normal levels, after the plasmid had been photoreactivated prior to transfection. Our results suggest that the CS cells repair all the dimers normally but do not repair the nondimer photoproducts. Our results also suggest that the XP cells do not repair all the dimers and do not repair all the nondimer photoproducts. We plan to continue these studies with cell lines from additional patients and to adapt the assay for measuring the effects of the ionizing-radiation-type of DNA damage which would make the assay suitable for testing the DNA-repair capacity of radiosensitive neurodegenerations such as Alzheimer's disease and Parkinson's disease.

In bacteria and yeast, specific DNA-damaging agents have been shown to induce DNA-repair genes. Transcripts of these genes are usually induced rapidly and are of low abundance. Our collaborator, Dr. Albert J. Fornace, Jr., attempted to identify DNA-repair genes in Chinese hamster cells on the basis of induction of the genes by UV radiation. Using a special hybridization subtraction procedure he had successfully used for heat shock-induced transcripts, he has succeeded in isolating 50 different Chinese hamster

cDNA clones which code for transcripts induced only 2- to 30-fold by UV-radiation. Some of these cDNA clones hybridize to UV-inducible transcripts in human fibroblast strains. We have prepared genomic DNA for Southern analysis from many human normal cell lines and from patients with DNA repair defects which make their cells hypersensitive to UV radiation (e.g., XP, CS). We plan to use the Fornace cDNA probes in Southern analysis (to detect mutational events in a gene) and Northern analysis (to measure qualitative and quantitative expression of the gene) in cells from patients with the UV-radiation-sensitive diseases, in an attempt to detect the mutations causing them. In further collaboration with Dr. Fornace, we plan to produce Chinese hamster and human cDNA libraries to transcripts induced by ionizing radiation and hydrogen peroxide. These cDNA probes will be used in Northern and Southern analyses of nucleic acids isolated from cells from the radiosensitive neuodegenerations in a attempt to identify the defects causing these diseases and their radiosensitivity.

Our findings of abnormal levels of UV-induced chromosomal aberrations in both CS and XP-group-C lymphoblastoid cell lines showed that the increase in such aberrations was not sufficient to cause skin cancer. There is a report in the literature that XP variants (who are unique among XP patients in having normal UV-induced unscheduled DNA synthesis) have a normal, instead of an abnormally high, level of UV-induced chromosomal aberrations. Since XP variants develop abnormal numbers of skin cancers, it would appear that abnormally high levels of induced chromosomal aberrations are not even necessary for the development of skin cancer. We propose to test this hypothesis by determining whether XP variants do, in fact, have only normal levels of UV-induced chromosomal aberrations. Our preliminary results indicate that XP variants have slightly abnormal levels of UV-induced chromosomal aberrations.

We are concluding our collaboration with John Nove on survival studies of fibroblast strains after their treatment with X-rays. We have found that Parkinson disease strains are not hypersensitive, and studies on Alzheimer disease strains are in progress.

With Dr. Miral Dizdaroglu and Dr. Ewa Gajewski, we are using capillary gas chromatography-mass spectrometry to detect various types of damage induced by ionizing radiation in DNA. We have completed a study of the effects of DNA conformation on the formation of 8,5'-cyclo-purine 2'-deoxyribonucleoside residues in isolated DNA irradiated in vitro. We are now studying the possibility of repair of these lesions in irradiated living cells.

We are performing autoradiographic studies of methylmethane sulfonate-induced unscheduled DNA repair synthesis in fibroblasts from patients with amyotrophic lateral sclerosis (ALS). These studies are being performed in an attempt to confirm a published report of others that ALS fibroblasts are deficient in repairing such induced DNA damage.

Publications:

Kinsella TJ, Dobson PP, Fornace Jr. AJ, Barrett SF, Ganges MB, Robbins JH. Alzheimer's disease fibroblasts have normal repair of N-methyl-N'-nitro-N-nitrosoguanidine-induced DNA damage determined by the alkaline elution technique, Biochem Biophys Res Commun 1987;149:355-361.

Seguin LR, Tarone RE, Liao K, Robbins JH. Ultraviolet light-induced chromosomal aberrations in cultured cells from Cockayne syndrome and complementation group C xeroderma pigmentosum patients: lack of correlation with cancer susceptibility, Am J Hum Genet 1988;42:468-475.

Dirksen M-L, Blakeley WF, Holwitt E, Dizdaroglu, M. Effect of DNA conformation on the hydroxyl radical-induced formation of 8,5'-cyclo-purine 2'-deoxyribonucleoside residues in DNA, Int J Radiat Biol 1988 (in press).

Robbins JH. Xeroderma pigmentosum: defective DNA repair causes skin cancer and neurodegeneration, JAMA 1988 (in press).

Ganges MB, Tarone RE, Jiang H, Hauser C, Robbins JH. Radiosensitive Down's syndrome lymphoblastoid lines have normal ionizing-radiation-induced inhibition of DNA synthesis. Mutation Res 1988 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03656-15 D

PERIOD COVERED

October 1, 1987 to September 30, 1988

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.I.: P.M. Steinert, Biologist, Dermatology Branch, NCI

COOPERATING UNITS *(if any)*

Experimental Pathology Branch, DCCP, NCI; Laboratory of Molecular Biology, DCBD, NCI; Laboratory of Physical Biology, NIDDK

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.5	5.5	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 biosynthesis, structure and function of the principal differentiation products of human and mouse epidermis are being studied. Keratin subunits polymerize in vitro into native-type intermediate filaments. Details of their structure are being investigated by use of: solid state NMR on isotopically-labeled filaments; transmission and scanning transmission electron microscopy of intact filaments or subfilamentous forms; optical diffraction and image analysis procedures; and limited proteolysis experiments to ascertain alignment of constituent subunits. Model structures generated by these methods are being computationally tested for compatibility with other physico-chemical data and amino acid sequence information on individual subunits. cDNA clones to keratins 1 and 10 are being used to characterize the number, organization and complexity of their genes isolated from cosmid libraries. The expression of the human genes is being studied by the production of transgenic mice produced from various constructs of these genes. cDNA clones are being used to isolate and characterize the genes for mouse and human filaggrin, which appear to be initially expressed as a very large polyprotein precursor. cDNA clones encoding a major cell envelop protein have been isolated and are being sequenced. Constructs of keratins, filaggrin and the cell envelop protein clones have been assembled with pGEM vectors for use in insitu hybridization experiments in order to study the expression of these proteins in epidermal keratinizing disorders.

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

Project Description

Other Professional Personnel:

J. Mack, Ph.D., Staff Fellow, Dermatology Branch, DCBD, NCI
J.A. Rothnagel, Ph.D., Visiting Fellow, Dermatology Branch, DCBD, NCI
L.J. McKinley-Grant, M.D., Guest Researcher, Dermatology Branch, DCBD, NCI
D. Hohl, Guest Researcher, Dermatology Branch, DCBD, NCI
W.W. Idler, Chemist, Dermatology Branch, DCBD, NCI
S.H. Yuspa, M.D., Branch Chief, Lab. of Cellular Carcinogenesis and Tumor Promotion, DCE, NCI
D.R. Roop, Ph.D., Senior Investigator, Lab. of Cellular Carcinogenesis and Tumor Promotion, DCE, NCI
A.C. Steven, Ph.D., Visiting Scientist, Lab. of Phys. Biol., NIAMS
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
R.D.B. Fraser, Division Chief, C.S.I.R.O., Protein Chemistry, Melbourne, Australia
J. Hainfeld, Professor, Department Biology, Brookhaven National Laboratory, Upton, New York
S. Lessin, Assistant Professor, Dermatology Department and Wistar Institute Philadelplhia, PA.

Major Findings:

The availability of complete amino acid sequences of several keratin chains has now led us to propose more advanced models of intermediate filament structure. Even though filaments are polymorphic, a major structural form consists of 8 4-chain molecules, arranged in an antiparallel mode. The structure of the 4-chain molecule is being examined by scanning transmission electron microscopy (with Dr. Hainfeld) using platinum-clathrates complexed onto cystine residues. In collaboration with Drs. A.C. Steven, David Parry and R.D.B. Fraser, we have constructed "surface-lattice" models of filament structure in which the rod domains of the protein chains form the basic filament core, and the variable end domains occupy peripheral positions. Limited proteolytic cleavage experiments have provided direct evidence in support of these structural models and of the precise alignment of the neighboring rod domains. In addition, scanning transmission electron microscopy is being used to explore the mass, structure and association of 4-chain units in an attempt to describe intermediate levels of filament substructure. However, the structural organization of the end domains is still uncertain. In the case of the keratins, we propose that their glycine-serine-rich sequences form omega-loop structures and interact with other cellular components including filaggrin and the cell envelop by H-bonding and disulfide cross-links. In collaboration with Dr. James Mack, these ideas are being tested by use of solid-state NMR of keratin filaments isotopically labeled with glycine (to study end domains) and leucine (to study the rod domain). Preliminary data suggests the glycine-rich domains have little or no structural order, which however, we expect should change when filaments are complexed with filaggrin.

In collaboration with R.D. Goldman (Northwestern University), mammalian nuclear lamin proteins have been isolated and characterized. Their chemical, immunological and biophysical properties clearly indicate that they are closely related to intermediate filaments, but are unable to form filaments in vitro: rather, they form tactoid structures in which their rod domains are loosely arranged in an open mesh-like network. This is very similar to their observed structure in vivo. Structural studies with Dr. David Parry have permitted the construction of models on how this network may be assembled.

Dr. Rothnagel has now isolated a major portion of the mouse filaggrin gene from a cosmid library, which contains 17 repeating units interspersed by one or two apparently randomly distributed tyrosine-rich linker sequences. He will construct a new cosmid library from genemic DNA restricted to completion with Dra I in an attempt to the full-length gene including upstream regulatory sequences. A cDNA isolate clone for human filaggrin has now been isolated from lambda gt11 expression library. This has been completely sequenced and demonstrates the presence of a 972 bp repeat different from mouse filaggrin. The full-length gene will also be isolated from a genomic library and human cosmid library. Dr. McKinley-Grant is using in situ hybridization techniques to study the expression of the human filaggrin in normal and abnormally keratinizing epidermis.

Dr. Hohl has isolated a cDNA clone encoding a cystine-rich cell envelop protein that is of considerable interest because its sequence is highly similar to the end domains of the keratin chains. He will use these probes to explore the role of this cell envelop protein in keratinizing disorders. Its full-length gene will be isolated from lambda genomic libraries.

Several cosmid clones carrying a large number of human keratin genes have been isolated. This work will enable for the first time detailed analyses of the structure, complexity, linkage and evolution of the keratin gene systems. One cosmid clone contains what appears to be two copies of the 67 kD keratin 1 gene, one of which was previously isolated by this laboratory. The keratin 10 gene has been isolated and mapped in a 25 kbp region without other nearby genes. These studies will also permit identification of regulatory sequences that control the expression of these genes during terminal differentiation. In collaboration with the Stuart Lessin, Wistar Institute, Philadelphia, PA, cDNA clones for both keratin 1 and keratin 10 have used to map their positions to chromosomes 10 and 17, respectively. Specific antibodies to the carboxylterminal end of the K1 and K10 proteins have been produced, and the cDNA clones have been subcloned for in situ hybridization experiments. Accordingly, experiments on the expression of these genes during normal and abnormal keratinization can now be initiated. In related experiments in collaboration with Dr. Dennis Roop, the keratin genes have been transfected into cells for examination of the regulatory sequences. Further experiments using microinjection into mouse oocytes, to generate transgenic mice containing the human keratin genes, are in progress to study regulation.

Publications:

Rothnagel JA, Mehrel T, Idler WW, Roop DR, Steinert PM. The gene for mouse epidermal filaggrin precursor, J Biol Chem 1987;262:15643-15648.

Steinert PM, Roop DR. The molecular and cellular biology of intermediate filaments, Ann Rev Biochem 1988;57:593-625.

Zhou X-M, Idler WW, Steven AC, Roop DR, Steinert PM. The complete sequence of the human intermediate filament chain keratin 10: subdomainal division and model for folding of end domain sequences, J Biol Chem 1988 (in press).

Steinert PM. The dynamic phosphorylation of the human intermediate filament keratin 1 chain, J Biol Chem 1988 (in press).

Lessin SR, Huebner K, Isobe M, Croce CM, Steinert PM. Chromosomal mapping of human keratin genes: evidence for non-linkage, J Invest Dermatol 1988 (in press).

Mack JW, Torchia DA, Steinert PM. Solid-state NMR studies on the dynamics and structure of mouse keratin intermediate filaments, Biochemistry 1988 (in press).

Steinert PM, Mack JW, Torchia D. Structural features of keratin intermediate filaments. In: Symposium of the Biology of Wool and Hair. 1988 (in press).

Roop DR, Hennings H, Mehrel T, Cheng C, Chung C, Rothnagel JA, Steinert PM, Yuspa SH. Sequential changes in gene expression during epidermal differentiation. In: Symposium on the Biology of Wool and Hair. 1988 (in press).

SUMMARY REPORT

LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY

October 1, 1987 to September 30, 1988

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, colon, ovarian, and lung carcinomas.

The Laboratory of Tumor Immunology and Biology was established in 1982. It is composed of six Sections: The Experimental Oncology Section (Dr. Jeffrey Schlom, Chief); 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 Cellular Biochemistry Section (Dr. Cho-Chung, Chief); The Cell Cycle Regulation Section (Dr. William Kidwell, Chief).

Monoclonal Antibodies Define Carcinoma Associated and 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 reagents for use in several aspects of the management of several human carcinomas. These include: detection of occult tumor cells, further defining the degree of differentiation of carcinoma cell populations, serum antigen assays, and radiolocalization of primary and metastatic carcinoma lesions in situ (and potentially therapy) using radiolabeled monoclonal immunoglobulins. These studies are divided into four areas of investigation: (I) The generation and characterization of an MAb that defines a novel tumor associated antigen (TAG72) (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 definition and characterization of breast and colon associated antigens and (IV) The generation and characterization of B72.3 second generation antibodies to further define the TAG-72 antigen and for potential utility in carcinoma management.

Characterization of Human-Tumor Associated Antigens

Human adenocarcinoma cells secrete and express on their cell surfaces high molecular weight, mucin-like glycoproteins that bear epitopes that are highly restricted to carcinoma cells. Monoclonal antibody B72.3, which has been extensively characterized as to reactivity with a variety of carcinomas versus normal tissues, reacts with such a mucin-like glycoprotein. The B72.3 reactive antigen, designated TAG (tumor-associated glycoprotein) -72, was partially

purified and used as immunogen to produce second generation anti TAG-72 antibodies. One of these second generation antibodies was selected to develop a procedure to purify preparative amounts of TAG-72. TAG-72, extracted from xenografted human colon carcinoma cells, was subjected to antibody affinity chromatography. A double determinant radioimmunoassay revealed a greater than 1000-fold purification with a greater than 10% yield. SDS-PAGE analysis of radiolabeled purified TAG-72 revealed a polydisperse, but apparently homogenous, high molecular weight glycoprotein. Amino acid analysis of the purified TAG-72 revealed a profile highly similar to that reported for other purified mucins. The purified TAG-72 has been partially deglycosylated with trifluoromethane sulfonic acid in preparation for amino acid sequence analysis.

Molecular Cloning and Analysis of Tumor-Associated Antigens

Studies carried out in our lab as well as others have demonstrated the presence of the TAG-72 and CEA tumor associated antigens in a high percentage of human carcinomas. Our studies have focused on understanding the role of these molecules in tumorigenesis as well as understanding the molecular mechanisms involved in the regulation of expression of these antigens. Specific probes for members of the CEA gene family will be used to study their expression in various tissues and cell lines and to examine the mechanism of regulation of CEA expression by interferons as well as other biological response modifiers. In addition, the gene encoding CEA will be cloned and transfected into mouse metastatic cell lines in order to obtain a mouse model for the study of anti-tumor agents.

Polyclonal antisera to deglycosylated TAG-72 and monoclonal antibodies to native TAG-72 will be used to obtain clones from cDNA libraries. Positive clones will be sequenced and used for analysis of TAG-72 expression in normal and tumor cell lines and tissues.

Monoclonal Antibodies to Detect Occult Carcinoma Cells

Monoclonal antibodies (MAbs) have been utilized with immunohistochemical methods for the (a) detection of occult carcinoma in surgical and cytology preparations, (b) phenotyping of malignant cell populations, (c) differentiation of histologic tumor types, and (d) identification of various cellular products. MAbs with selective reactivity against tumor associated antigens have specifically been adapted for use with cytologic preparations including cytospins, membranes, and fine needle aspiration biopsies (FNAB) for the detection and differentiation of carcinomas from benign cell types and other cancerous lesions.

Augmentation of Tumor Antigen Expression

The ability to effectively target radionuclide conjugated monoclonal antibodies (MAb) to human tumor lesions depends, in part, on the degree to which the tumor cell population expresses the reactive carcinoma-associated antigens. Studies have shown that human tumor cells can intrinsically modulate their antigenic phenotype in response to cell-cycle kinetics, clonal variability, cell-to-cell interactions and other cellular factors. The resultant variability in the antigenic phenotype contribute to the antigenic heterogeneity present for most tumor antigens. We have established that certain biological response modifiers, specificaly human recombinant interferons, can upregulate some MAb-defined tumor antigens as well as induce the expression of class I and class II major histocompatibility antigens. Utilizing primarily human breast and colorectal tumor cell lines, recombinant leukocyte (clone A), the serine-sub-

stituted recombinant beta and recombinant gamma interferon have been shown to increase CEA and TAG-72 expression. Subsequent studies have also shown that the in vivo administration of the recombinant leukocyte (clone A) interferon can effectively increase CEA and the B6.2 reactive 90KD antigen in human extracts. In addition, the interferon increased in the MAb defined tumor antigens was also accompanied with an enhanced targeting of the radioconjugated MAb. These experimental studies clearly indicate that the antigenic phenotype of a human tumor cell population can be significantly altered following the administration of a recombinant human interferon. This effective activity of these biological response modifiers may be utilized as adjuvants in a clinical setting for the detection and/or therapy of occult lesions by conjugated MAbs.

Heterobifunctional Antibodies in Tumor Targeting

The anti-tumor activity of T-cells, NK cells, and macrophages can be augmented by lymphokines and cytokines, monoclonal antibodies (Mabs) against tumor antigens, and combinations of MAbs and effector cell activators. Heteroantibodies with dual specificity were constructed by chemically cross-linking Mab against the human T-cell CD3 antigen with antibodies recognizing surface antigens on colon and ovarian tumors. Utilizing normal human peripheral blood lymphocytes, it was shown that these heteroantibodies, and in some cases, monomeric anti-tumor Mab, potentiated the killing of cultured tumor cells. In addition, heteroantibody-directed cytotoxicity was enhanced by effector cell activation with either IL-2 or anti-CD3 Mab. Thus, heteroantibodies may provide a new immunotherapeutic strategy for targeting cytotoxic immune cells to tumors.

Immunoassays for Human Carcinoma Associated Antigens

Immunoassays for the detection of human carcinoma associated antigens and assays needed to support the clinical use of monoclonal antibodies have been developed. These assays are being used to examine body fluids, such as serum, ascities, and pleural effusions for the presence of tumor associated antigens. A competitive radioimmunoassays (RIA) has been developed to detect a tumor associated glycoprotein (TAG-72) identified by MAb B72.3. The competitive RIA and a second RIA, a double determinant immunoassay, detecting the same antigen, have been used to examine sera from patients with colorectal carcinomas, and other malignancies as well as normal sera. A mean of approximately 2 units/ml of TAG-72 was found in normal sera. Approximately 60% of patients with colorectal carcinomas have elevated (>10 U/ml) levels of TAG-72 in their serum. Comparison of TAG-72 levels in sera with assays to detect other antigens also recognized by monoclonal antibodies 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 RIAs.

Immunoassays are being developed to support clinical studies for evaluating the efficacy of MAbs to detect carcinomas as well as deliver therapeutic agents. Assays for the detection of circulating immune complexes, both antigen-antibody and antibody-antibody complexes, using high-performance liquid chromotography have been developed. Assays for the evaluation of the immunoreactivity and form of the injected MAb B72.3, as well as assays to examine the development of human anti-mouse antibodies have been developed.

Localization and Therapy Using Labeled Monoclonal Antibodies: Model Systems

Monoclonal antibody (MAb) B72.3 binds to many human epithelial malignancies including breast, colon, ovarian and lung cancer. Radioiodinated B72.3 IgG can selectively bind to human colorectal carcinomas grown in athymic mice; the 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. B72.3 IgG was labeled with In-111 using four chelating agents: 1-(p-isothiocyanato-benzyl)-DTPA (SCN-Bz-DTPA), MA-DTPA, CA-DTPA, and the SCN-Bz derivative of EDTA. Comparative biodistribution studies demonstrated that tumor uptake of radiolabel was very similar between the chelates (30% ID/g), however, a progressive accumulation of activity in the abdominal organs, predominantly in the liver, was seen with the MA-DTPA, CA-DTPA and SCN-Bz-EDTA chelate-antibody complexes. This uptake was very prominent with these chelate-MAb complexes but was virtually absent in the mice injected with B72.3SCN-Bz-DTPA.

The high degree of selective binding of this MAb has led to the investigation of its potential as a radioimmunotherapeutic agent. Athymic mice bearing human colon carcinoma xenografts were injected with B72.3 IgG to assess the effect of the radiolabeled MAb on the tumor growth as well as potential toxic side effects in vital organs. In mice treated with the I-131 B72.3 IgG, a marked inhibition of the growth of the human colon carcinoma xenografts was noted. Toxicity was readily evident in the mice injected with the high-dose regimen (500 μCi), with confirmed bone marrow aplasia that proved lethal for 2 of 10 animals. Lower doses (300 μCi) resulted in a bone marrow suppression of approx. 50% of the cells, which proved to be non-lethal. The tumors in the treated mice showed extensive necrosis caused by the lethal dose of I-131-B72.3 that irreversibly damaged the cells.

Parameters Involved in the Tumor Targeting of Monoclonal Antibodies

One of the problems observed in studies using radiolabeled MAbs for detection of human tumors is the relatively low percent of the injected dose of monoclonal antibody (MAb) per gram of tumor. Recent studies in *in vivo* model systems for melanoma and hepatoma, and clinical trials involving hepatoma patients have suggested enhanced deposition of radiolabeled antibody in tumors pretreated with external irradiation. One major goal of this project is to identify parameters that optimize binding of MAbs to carcinoma cells *in vivo*. We have recently initiated studies to determine the effect of exposure of carcinomas to external irradiation prior to localization of tumors by MAb conjugates. These studies include evaluation of the effect of external irradiation on (a) growth of carcinoma xenografts, (b) cellular integrity and the expression of TAG-72 at the cellular level, and, (c) binding of MAb B72.3 to carcinoma xenografts.

One approach to the treatment of patients with peritoneal carcinoma may be direct infusion into the peritoneal cavity of radiolabeled antibody conjugates. In comparison to intravenous MAb administration, this method may improve the percent of the injected MAb dose per gram of tumor and may lead to less radiolabeled antibody in the bone marrow and, therefore, less toxicity to the patient. The second major goal of this project is to develop an *in vivo* model system to study immunotherapy of human carcinoma in the peritoneal cavity. These studies will include (a) determination of the animal species eliciting a pattern of clearance of MAb from the blood most comparable to that observed in the human, (b) development of a human peritoneal carcinoma xenograft in an animal model, and

(c) determination of the utility of intraperitoneally administered MAb conjugates for therapy of carcinoma.

Clinical Trials with Radiolabeled 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 and several other collaborating institutions to detect and localize carcinoma lesions using radiolabeled monoclonal antibody (MAb) B72.3. Parameters that are being 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) type of carcinoma (colorectal, ovarian, lung, breast) (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 development of human anti-murine Ig antibodies; (h) metabolism of MAb. 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).

Hormones and Growth Factors in Development of Mammary Glands & Tumorigenesis

This project is designed to determine the role of hormones and growth factors in the development and differentiation of normal mammary gland and in the development, growth, and maintenance of mammary tumors. Studies include: 1) examining the roles of epidermal growth factor and mammary gland-derived growth factors in lobuloalveolar 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) examining the effect of sialadenectomy on mammary tumor incidence and growth in mice as well as the production of preneoplastic hyperplastic alveolar nodules; and 4) defining the hormone receptor and response status of the NOG-8 mouse mammary epithelial cell line.

Regulation of Lactogenic Hormone Receptors in Mammary Tissue

This project is designed to evaluate the nature of lactogenic hormone receptors and the factors (including other hormones) that affect the 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; 3) characterization of the nature of the interaction of Tamoxifen with membrane-bound receptors related to the lactogen receptor; and 4) definition of the relationship of monoclonal antibody B6.2 to human lactogenic hormone receptors.

CELLULAR AND MOLECULAR PHYSIOLOGY SECTION (Dr. Herbert L. Cooper, Chief)

Studies have continued on the role of derangements in tropomysin (TM) expression and utilization in neoplastic transformation. Neoplastic transformation secondary to expression of retroviral oncogenes is correlated with the production of TM-deficient, structurally defective, microfilaments in both fibroblasts and epithelial cells. This may be a basic structural and functional lesion which is conducive to expression of components of the transformed phenotype. The derangement results either from suppression of synthesis and utilization of TM (fibroblasts), or from relative overexpression of actin (epithelial cells). TM suppression in fibroblasts transformed by retroviral oncogenes may be due to the action of alpha-transforming growth factor produced as a consequence of oncogene expression. TM expression was also studied in a panel of established human breast cancer lines. Consistent abnormalities in tropomysin expression were observed in the tumor cell lines, suggesting that derangement of TM expression may be a frequent event in human mammary neoplasia.

Studies have continued on the unique 18.4K, pI 5.9 phosphoprotein, prosolin (formerly called ppl7), a major cytosolic protein which undergoes rapid phosphorylation in HL60 promyelocytic leukemia cells when their growth is inhibited and they are induced to differentiate in response to treatment with phorbol ester (TPA). The expression and phosphorylation of prosolin in human peripheral lymphocytes (PBL) was investigated. Prosolin was identified in PBL, and its expression was found to be correlated with the S-phase of the cell cycle. In proliferating PBL prosolin was a major cytosolic component, comprising 0.5% of total cytosolic protein, of which 25% was found in 2 phosphorylated forms. TPA treatment of proliferaing PBL caused phosphorylation of about 2/3 of pre-existing unphosphorylated prosolin within 1 hr followed immediately by an abrupt cessation of DNA synthesis. Phosphorylation of prosolin may be an initiating event in the antiproliferative response to TPA. The availability of prosolin only during S-phase may provide a regulatory mechanism by which naturally occuring cytokines may terminate lymphocyte growth, through activation of antiproliferative processes linked to phosphorylation of prosolin, without inhibiting activation of G_0 or G_1 cells.

BIOCHEMISTRY OF ONCOGENES SECTION (Dr. Robert Bassin, Chief)

Studies in the Biochemistry of Oncogenes Section are focussed on experimental systems designed, ultimately, to uncover mechanisms by which oncogenes transform cells both *in vitro* and *in vivo*. An essential part of this effort concerns the identification and occurrence of transforming growth factors, studies on their mechanism of action, and their functional relationships to transforming oncogenes. Both epithelial and mesenchymal models are included.

Primary DMBA- and NMU-induced rat mammary carcinomas contain a specific 4.8 Kb alpha TGF message. *In vitro*, the NOG-8 mouse mammary epithelial cell line transfected with a glucocorticoid-sensitive MMTV LTR:*ras* molecular chimera produce the *ras* gene product, p21, and express alpha TGF mRNA within a few hours after addition of dexamethasone. Overexpression of the human TGF alpha gene introduced into NOG-8 cells is sufficient to transform these cells both *in vitro* and *in vivo*.

Elevated levels of alpha TGF and its message can be detected in 65% of primary

human breast tumors, and the human mammary tumor line MCF-7 can be growth inhibited following treatment with either anti-TGF or anti-EGF receptor antibodies.

The mechanism of transformation of cells by oncogenes has also been approached by isolating cells which are resistant to transformation by specific oncogenes. Our original set of revertants, that are resistant to transformation by ras and certain other oncogenes, have been recently studied with respect to monovalent cation transport. This work is based on the original finding that ouabain, a specific inhibitor of Na, K ATPase, is highly toxic for ras-transformed cells as opposed to control NIH/3T3 cells. Revertants lose their sensitivity to ouabain. We have recently found that there is indeed a transformation-related alteration in ion transport in a variety of cells transformed by ras, including human and murine, epithelial and fibroblastic cells. A clear change (about 2-fold) in the activity of the Na/H exchanger (antiporter) can be seen in ras-transformed cells under defined experimental conditions. The Na/H exchanger is stimulated by exogenous alpha TGF and is known to play a part in the alkalinization of cells prior to cell division.

New methods useful for the generation and isolation of revertant cells resistant to transformation to other oncogenes are being developed. The amino acid analog cis-hydroxyproline has been found to be useful in the isolation of revertants, and the properties of CHP-resistant revertants isolated from ras-transformed cells are under current investigation.

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 descriptin 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. In addition, the Oncogenetics Section collaborates with the Experimental Oncology Section to develop, using recombinant DNA technology, derivatives of monoclonal antibodies against tumor associated antigens which are to be used in clinical therapy trials. During the past year the Oncogenetics Section has focussed its efforts in four areas: (1) isolation and characterization of recombinant cDNA clones of the mouse mammary tumor int-3 gene; (2) determine the effect of the host genetic background on the frequency of MMTV induced int gene activation in mouse mammary tumors, (3) identification of frequently altered proto-oncogenes in primary human breast tumors and (4) obtain recombinant clones of MAb heavy and light chain gene to develop chimeric mouse human derivative MAbs.

Mammary Tumorigenesis in Inbred and Feral Mice

We have previously described a common integration site (designated int-3) for MMTV in virus-induced mouse mammary tumors. MMTV integration at this locus

activates the expression of a 6.6- and 2.4-kb species of RNA. These RNA species correspond to flanking cellular genomic sequences that are located 5' and 3', respectively, of the integrated viral genome. We are currently analyzing cDNA recombinant clones of the 2.4-kb species of int-3 RNA. In other studies, we have shown that there is a significant difference in the frequency with which MMTV (C3H) integrates at int-1 in BALB/cfC3H compared with C3H mammary tumors. Similarly, there is a significant difference in the frequency of MMTV (RIII) integration events at int-2 in BALB/cfRIII compared with RIII mammary tumors. These results suggest that the inbreeding program for high tumor incidence in C3H and RIII mouse strains has selected for host genetic mutations that affect the frequency of int gene activation. To begin to dissect at a genetic level the mutational events resulting in the development of preneoplastic and malignant mammary epithelial phenotypes, we have developed several mammary hyperplastic outgrowth lines (HOGs) in CZECHII V^+ mice. These HOGs and primary mammary epithelia are currently being used as targets for retroviral vectors containing the int genes and other oncogenes.

The Identification and Characterization of Human Genes Associated with Neoplasia

We have continued our efforts to identify and characterize frequent genetic changes associated with primary human breast tumor DNAs. The int-2 gene is related to the fibroblast growth factor gene family. It is frequently activated by mouse mammary tumor virus-induced insertional mutagenesis in mouse mammary tumors. We have found that int-2 is amplified 2- to 15-fold in 16% of human breast tumors (n=118). This genetic alteration has a highly significant association ($P \leq 2 \times 10^{-6}$) with patients who subsequently developed a local recurrence or distal metastasis. The c-erbB-2 gene is related to the gene encoding the epidermal growth factor (EGF) receptor. We found that 10% of the breast tumor DNA contain an amplification of c-erbB-2. In contrast to one published report from another laboratory, we did not find a significant association between this mutation and the patients' prognosis.

CELLULAR BIOCHEMISTRY SECTION (Dr. Yoon Sang Cho-Chung, Chief)

The Cellular Biochemistry Section studies the mechanism of cell growth and differentiation by cyclic AMP (cAMP). It is anticipated that the studies outlined below by the use of new derivatives of cAMP, site-selective cAMP analogs, which far exceed in their effect the parent cAMP or the previously studied cAMP analogs, may lead to: (1) a better understanding of the regulatory mechanisms of cell growth and differentiation, (2) a clearer definition of derangement of cAMP-effector function in neoplastic transformation and progression, and (3) improved management of human cancers by providing the non-toxic biological compounds (site-selective-cAMP analogs) as antineoplastic.

Our studies being conducted in three projects are summarized below:

Cyclic AMP (cAMP) in Growth Control of Neoplasia

The physiologic role of cAMP in the growth control of a spectrum of human cancer lines, including leukemic lines, and v-rasH oncogene-transformed NIH/3T3 cells is demonstrated by the use of site-selective cAMP analogs. These cAMP analogs, which can select either of the two known cAMP binding sites of the cAMP receptor protein, induce potent growth inhibition, phenotypic change, and differentiation (leukemic cells) of cancer cells at micro-

molar concentrations with no sign of cytotoxicity. The growth inhibition parallels selective modulation of cAMP-dependent protein kinase isozymes, type I versus type II, and suppression of cellular proto-oncogene expression. Site-selective cAMP analogs thus provide new biological tools for investigating cell proliferation and differentiation and also for the improved managment of human cancers.

cAMP Receptor Protein in Gene Transcription

Recent evidence suggests a direct action of cAMP on mamalian genes in which the cAMP receptor protein, the regulatory subunit of cAMP-dependent protein kinase might interact directly with target genes. Since cAMP-dependent protein kinase is exclusively present in the cytoplasm, any nuclear function of the protein kinase must require its nuclear translocation. It was found that the ras gene suppression, growth inhibition, and phenotypic reversion of Ha-MuSv-transformed NIH/3T3 cells by site-selective cAMP analogs correlated with the nuclear translocation of RII cAMP receptor protein which was detected within 30 minutes after the analog treatment. The goal of this study is to provide direct evidence of interaction between the cAMP consensus sequences and cAMP receptor protein and/or a phosphoprotein substrate of protein kinase.

Cellular Proto-Oncogene Expression and Malignancy

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 the study of more than 200 human primary breast carcinomas, we have observed elevated expression (2-10 fold over that of benign breast tumors and normal breast) of c-rasH in 74% of tumors. Moreover, the degree of p21 (c-ras gene product) elevation correlated with the progression and poor prognosis of the disease. Whereas, an amplified or re-arranged c-rasH gene has not been detected in human mammary carcinoma. The goal of this study is to elucidate the mechanism of oncogene involvement in the neoplastic transformation and progression.

CELL CYCLE REGULATION SECTION (Dr. William R. Kidwell, Chief)

Direct and indirect evidence suggests that positive and negative growth regulatory factors are secreted by normal cells and these factors, in turn, act on the producing cells. Furthermore, alterations in the production of these growth modulating factors is thought to play role(s) in the loss of growth control as cells become cancerous. Our objective has been to assess such a possibility by studying the production of growth modulators by normal and neoplastic mammary epithelium from rodent and human tissues and fluids. To date we have identified four such activities, two stimulatory and two inhibitory factors. One stimulatory factor purified from human mammary tumors and human milk is Mammary Derived Growth Factor I (MDGFI). The factor is a 62 Kd protein that acts via cell membrane receptors. It stimulates mammary cell growth at pMolar levels. MDGF1 production is about three times higher in tumors than in normal human mammary epithelium. MDGFI has been purified to apparent homogeneity and its N-terminal sequence determined to be Ile-Pro-Val-Lys-Gln-Ala-Val-His-GlyGln-Phe-Leu-Leu-Pro-Lys-Gln. A computer search failed to find any protein with a significant homology to the N-terminal sequence of MDGFI. MDGFI may thus represent a growth

regulatory factor whose overproduction favors rapid breast cancer cell growth. A second growth promoting factor made by human and rodent breast cells is Transforming Growth Factor Alpha (TGFα). We have found that this factor is produced by normal cells and in some breast tumors. Production appears to be regulated by estrogenic hormones, especially in rodents. Estradiol 17-β , in physiological amounts, enhances production of TGFα and its mRNA. In rodents (and possibly in humans) production of TGFα appears to be correlated with the presence of estrogen and progesterone receptors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05190-08 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Jeffrey Schlom	Chief	LTIB, DCBD, NCI
Patricia Horan Hand	Chemist	LTIB, DCBD, NCI
Jean Simpson	Medical Staff Fellow	LTIB, DCBD, NCI
Ricardo Parker	BTP	LTIB, DCBD, NCI
Masahide Kuroki	Visiting Associate	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

P. Noguchi, Bureau of Biologics, FDA
W. Johnston, Dept. of Pathology, Duke Univ.
F. Gorstein, Dept. Pathology, Vanderbilt University

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.3	4.3	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 several human carcinomas. These include: detection of occult tumor cells, further defining the degree of differentiation of carcinoma cell populations, serum antigen assays, and radiolocalization of primary and metastatic carcinoma lesions in situ (and potentially therapy) using radiolabeled monoclonal immunoglobulins. 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 definition and characterization of breast and colon associated antigens and (IV) The generation and characterization of B72.3 second generation antibodies to further define the TAG-72 antigen and for potential utility in carcinoma management.

Major Findings:

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 antigen assays; and radiolocalization of primary and metastatic carcinoma lesions in situ (and potentially therapy) using radiolabeled monoclonal immunogloubins 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 eiptopes on carcinoembryonic antigen (CEA) which are differentially expressed among carcinoma cell populations; (III) The definition and characterization of breast and colon associated antigens and (IV) The generation and characterization of B72.3 second generation antibodies to further define the TAG-72 antigen and for potential utility in carcinoma management.

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 ovarian, 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 colon, breast, and ovarian carcinomas. 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.

Publications

Muraro R, Wunderlich D, Thor A, Cunningham R, Noguchi P, and Schlom J: Immunological characterization of a novel human colon associated antigen (CAA) by a monoclonal antibody: Int. J. Cancer 39, 34-44, 1987.

Schlom J: Future prospects in breast cancer research and management. M. Lippman, A. Lichter, D. Danforth, (Eds). The Diagnosis and Management of Breast Cancer. W. B. Saunders Company, Philadelphia, PA, 1988, pp. 549-556.

Thor A, Muraro R, Gorstein F, Ohuchi N, Viglione M, Szpak CA, Johnston WW, and Schlom J: Adjunct to the diagnostic distinction between adenocarcinomas of the ovary and the colon utilizing a monoclonal antibody (COL4) with restricted carcinoembryonic antigen reactivity. Cancer Research 47: 505-512, 1987.

Kufe DW, Hayes DF, Abe M, Ohno T, and Schlom J. Monoclonal antibody assays for breast cancer: H. C. Kupchik (Ed.) In Vitro Diagnosis of Human Tumors Using Monoclonal Antibodies, New York, Marcel Dekker Inc., pp. 67100, 1988.

Horan Hand P, Vilasi V, Caruso A, and Schlom J: Absolute values of ras p21 defined by direct binding liquid competition radioimmunoassays. Biochimica et Biophysica Acta. 908: 131-142, 1987.

Thor A, Viglione MJ, Muraro R, Ohuchi N, Schlom J. and Gorstein F.: Monoclonal antibody B72.3 reactivity with human endometrium: a study of normal and malignant tissues. Int. J. Gynecological Pathology 6: 235-247, 1987.

Horan Hand P, Vilasi V, Thor A, Ohuchi N, and Schlom J.: Quantitation of Harvey ras p21 Enhanced Expression in Human Breast and Colon Carcinomas. J. Natl. Cancer Inst 79: 59-65, 1987.

Lan MS, Bast RC, Colnaghi MI, Knapp RC, Colcher D, Schlom J, and Metzgar RS: Co-Expression of human cancer-associated epitopes on mucin molecules. Int. J. Cancer. 39: 68-72, 1987.

Ohuchi N, Horan Hand P, Merlo G, Fujita J, Mariani-Costantini R, Thor A, Nose M, Callahan R, and Schlom J: Enhanced expression of c-Haras p21 in stomach adenocarcinomas defined by immunoassays using monoclonal antibodies and in situ hybridization. Cancer Research. 47: 1413-1420, 1987.

Johnston WW, Szpak CA, Thor A, Simpson JF, and Schlom J: Applications of immunocytochemistry to clinical cytology. Cancer Investigation 5(6), 593-611, 1987.

Ohuchi N, Wunderlich D, Fujita J, Colcher D, Muraro R, Nose M, and Schlom J: Differential expression of carcinoembryonic antigen in early gastric adenocarcinomas versus benign gastric lesions defined by monoclonal antibodies reactive with restricted antigen epitopes. Cancer Research 47: 3665-71, 1987.

Ohuchi N, Simpson J, Colcher D, and Schlom J: Complementation of anti CEA and anti-TAG-72 monoclonal antibodies in reactivity to human gastric adenocarcinomas. Int. J. Cancer 40: 726-733, 1987.

Czerniak B, Herz F, Koss LG, and Schlom J: ras oncogene p21 as tumor marker in cytodiagnosis of gastric and colonic carcinomas. Cancer. 60: 2432-2436, 1987.

Johnston WW, Szpak CA, Thor A, Simpson J, and Schlom J: Antibodies to tumor-associated antigens: Applications in clinical cytology. In Wied, Koss, and Reagan (Eds.): 5th Edition of Compendium on Diagnostic Cytology, International Academy of Cytology, pp. 567-578,

Colcher D, Simpson J, Ohuchi N, Carrasquillo JA, Reynolds JC, Larson SM, and Schlom J: Potential use of monoclonal antibody B72.3 for the diagnosis and management of gastrointestinal malignancies. In: K. Sugahara (Ed.) New Trends of Gastroenterology, 1987 pp. 153-165.

Vick WW, Bigner SH, Wikstrand CJ, Bullard DE, Kemshead J, Coakham H, Schlom J, Johnston WW, and Bigner DD: The use of a panel of monoclonal antibodies in the evaluation of cytologic specimens from the central nervous system. Acta Cytol 31: 815-824, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09009-07 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters 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	Cancer Expert	LTIB, DCBD, NCI
Fiorella Guadagni	Visiting Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

D. S. Pestka, UMDNJ - Robert Wood Johnson Medical School, Piscataway, NJ
E. Bordon, U. Wisconsin Med. Center, Madison, Wisconsin
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 20892

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.)*

The ability to effectively target radionuclide conjugated monoclonal antibodies (MAb) to human tumor lesions depends, in part, on the degree to which the tumor cell population expresses the reactive carcinoma-associated antigens. Studies have shown that human tumor cells can intrinsically modulate their antigenic phenotype in response to cell-cycle kinetics, clonal variability, cell-to-cell interactions and other cellular factors. The resultant variability in the antigenic phenotype contribute to the antigenic heterogeneity present for most tumor antigens. We have established that certain biological response modifiers, specificaly human recombinant interferons, can upregulate some MAb-defined tumor antigens as well as induce the expression of class I and class II major histocompatibility antigens. Utilizing primarily human breast and colorectal tumor cell lines, recombinant leukocyte (clone A), the serine-substituted recombinant beta and recombinant gamma interferon have been shown to increase CEA and TAG-72 expression. Subsequent studies have also shown that the in vivo administration of the recombinant leukocyte (clone A) interferon can effectively increase CEA and the B6.2 reactive 90KD antigen in human extracts. In addition, the interferon increased in the MAb defined tumor antigens was also accompanied with an enhanced targeting of the radioconjugated MAb. These experimental studies clearly indicate that the antigenic phenotype of a human tumor cell population can be significantly altered following the administration of a recombinant human interferon. This effective activity of these biological response modifiers may be utilized as adjuvants in a clinical setting for the detection and/or therapy of occult lesions by conjugated MAbs.

GPO 914-918

Major Findings

Human Hu-IFN-α is a multigene family. We compared Hu-IFN- αA,B,C,D, F,I,J and K for their abilities to growth inhibit human breast carcinoma cells as well as to augment the expression of surface antigens. A high degree of heterogeneity with respect to these biological parameters was found among the different Hu-IFN-α species. The A and B species were most potent in cell growth inhibition and augmenting MAb binding to surface antigens such as HLA, carcinoembryonic antigen (CEA) and a high-molecular weight tumor-associated glycoprotein, termed TAG-72. The effects of recombinant human leukocyte (clone A) interferon (Hu-IFN-αA) were investigated on the expression of MAb-defined tumor antigens expressed on human mammary and colon carcinomas. Hu-IFN-αA treatment substantially increased the localization of radiolabeled MAb B6.2-F(ab')$_2$ to the transplantable Clouser human mammary carcinoma, as well as to the moderately differentiated human colon xenograft WiDr, when grown as subcutaneous tumors in athymic mice. In contrast, human tumor cell lines (i.e., LS174T, A375, etc.) that were unresponsive to the antigen-augmenting ability of Hu-IFN αA in vitro were also unresponsive in vivo, indicating a possible method of screening carcinoma cell populations for subsequent interferon adjuvant therapy prior to MAb administration. The method of delivery of Hu-IFN- αA was also studied. The intramuscular route resulted in a 3-4 h plasma half-life for Hu-IFN-α A. The administration of Hu-IFN-α A via an osmotic pump resulted in a stable circulating plasma titer of 400-800 antiviral units/ml for 7 days. Utilizing delivery of rHu-IFNαA by the constant infusion route, it was found that the increase in localization of 125I-B6.2-F(ab')2 was dependent on (1) the length of time of treatment with the interferon and (2) the circulating plasma Hu-IFN-αA levels. These results thus provide information useful for subsequent studies to determine the potential efficacy of adjuvant interferon treatment for MAb targeted tumor diagnosis and treatment. In contrast, the D and J species were virtually inactive in altering the expression of these or any other surface antigen both in vito and in vivo. Hu-IFN-αD and - αJ did interact with the interferon receptor by their ability to interfere with the rHu-IFN- αA induced antigen augmentation on the MCF-7 cell surface. In conclusion, the Hu-IFN-α family exerts a wide range of biological actions. These findings are important for identifying those interferon species that are capable of augmenting tumor antigen expression, and thus, may be earmarked for consideration in further study of the enhancement of MAb binding to human carcinoma cell populations.

Publications

Tran R, Horan Hand P, Greiner JW, Pestka S and Schlom J: Enhancement of surface antigen expression on human breast carcinoma cells by recombinant human interferons. J. Interferon Res. 7:75-88, 1988.

Greiner JW, Fisher PB, Pestka S, and Schlom J: Differential effects of recombinant human leukocyte interferons on cell surface antigen expression. Cancer Res. 46: 4984-4990, 1986.

Greiner JW, Pestka S, Fisher PB, and Schlom J: Augmentation of surface antigens on human carcinoma cells by recombinant leukocyte interferon. In The Interferon System: A Current Review, S. Baron, M.D. (Ed.), University of Texas Press, Austin, TX, 1986. (In press).

Schlom J, Colcher D, Horan Hand P, Thor A, Greiner J, and Weeks, MO: Potential clinical applications of monoclonal antibodies in colorectal and mammary cancer. In: Monoclonal Antibodies in the Diagnosis and Therapy of Cancer. Jack A. Roth (Ed.), Futura Publishing Co, Inc., Mount Kisco, NY, 1986, 1-27.

Greiner JW, Guadagni F, Noguchi P, Pestka S, Colcher D, Fisher PB, and Schlom J: Recombinant interferon enhances monoclonal antibody-targeting of carcinoma lesions in vivo Science 235: 895-898, 1987.

Guadagni F, J Schlom, and JW Greiner. Differential increases of human antigen expression by human leukocyte interferons. In Winkelhake, J.L. and Holcenberg, J.S. (Eds.), The Pharmacology and Toxicology of Proteins, UCLA Symposia on Molecular and Cellular Biology, New York, Alan R. Liss, Inc., vol. 51 pp. 287-298, 1987.

Greiner J.W., J. Schlom, S. Pestka, P. Giacomini, M. Kusama, S. Ferrone, and P.B. Fisher. Modulating of tumor-associated antigen expression and shedding by human recombinant DNA leukocyte and fibroblast interferons. Pharmacol. Therapeut. 31: 3, 1987.

Greiner JW, P. Horan Hand, D. Colcher, M. Weeks, A. Thor, P. Noguchi, S. Pestka, and J. Schlom. Modulation of human tumor antigen expression. J. Lab. Clin. Med. 109: 244, 1987.

Guadagni, F., J.W. Greiner, S. Pothen, S. Pestka, and J. Schlom. Enhanced in vivo monoclonal antibody localization to human carcinomas in athymic mice after recombinant interferon treatment. Cancer Immunol. Immunotherapy (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05233-07 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Immunoassays 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
Patrizia Ferrone	Visiting Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Drs. J. Carrasquillo and J. Reynolds, Nuclear Med., NIH
Dr. W. Johnston, Dept. Pathology, Duke Univ. School of Medicine

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

A

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

Immunoassays for the detection of human carcinoma associated antigens and assays needed to support the clinical use of monoclonal antibodies have been developed. These assays are being used to examine body fluids, such as serum, ascities, and pleural effusions for the presence of tumor associated antigens. A competitive radioimmunoassays (RIA) has been developed to detect a tumor associated glycoprotein (TAG-72) identified by MAb B72.3. The competitive RIA and a second RIA, a double determinant immunoassay, detecting the same antigen, have been used to examine sera from patients with colorectal carcinomas, and other malignancies as well as normal sera. A mean of approximately 2 units/ml of TAG-72 was found in normal sera. Approximately 60% of patients with colorectal carcinomas have elevated (>10 U/ml) levels of TAG-72 in their serum. Comparison of TAG-72 levels in sera with assays to detect other antigens also recognized by monoclonal antibodies 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 RIAs.

Immunoassays are being developed to support clinical studies for evaluating the efficacy of MAbs to detect carcinomas as well as deliver therapeutic agents. Assays for the detection of circulating immune complexes, both antigen-antibody and antibody-antibody complexes, using high-performance liquid chromotography have been developed. Assays for the evaluation of the immunoreactivity and form of the injected MAb B72.3, as well as assays to examine the development of human anti-mouse antibodies have been developed.

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

Major Findings

The purpose of these studies is to identify and characterize tumor associated antigens (TAAs) detected by monoclonal antibodies that bind human carcinomas and to determine if these monoclonal antibodies bind to antigens distinct from known TAAs as well as 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. The distribution of TAG-72 in sera from patients with colorectal carcinomas and other malignancies was examined. In a competitive radioimmunoassay and a double determinant RIA using a MAb to TAG-72 bound to a solid matrix to quantitate TAG-72 in human serum the mean TAG-72 concentration in 1,099 serum samples from healthy blood donors the mean value obtained was 1.83$\pm$ 2.03 (SD) units/ml. If the upper limit of normal was set at 10 U/ml of serum, a value including 99% of healthy blood donors, only 4 of 101 serum samples (4%) from patients with benign disease were elevated, whereas 15 of 26 (58%) and 14 of 25 (56%) of rectal and colon carcinoma patient sera, respectively, were positive. Serum samples from 84 benign colorectal disease cases were examined; of these 0 of 28 (0%) colorectal adenoma, 1 of 39 (3%) ulcerative protocolitis, 0 of 15 (0%) diverticulosis, and 0 of 2 (0%) irritable bowel disease sera contained more than 10 U/ml TAG-72. At a reference value of 20 U/ml, 0 of 101 (0%) benign disease and 2 of 1,060 (0.2%) blood donor sera had elevated values, whereas 10 of 26 (38%) and 9 of 25 (36%) rectal and colon patient sera, respectively, remained positive. The majority of patients with pancreatic and ovarian cancer, and a significant fraction of stomach cancer patient sera, also contained elevated levels of TAG-72.

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 commercial available MAb RIAs.

This assay is currently being used to evaluate sera of patients with other malignancies as well as other biological fluids such as pleural effusions and ascitic fluid for the presence of TAG-72. The assay is also being used to study patients in the clinical trial for the *in vivo* localization of malignancy using radiolabeled B72.3. Sera is being examined to determine if there is any correlation between TAG-72 levels in a patient's serum and the efficacy of the radiolabeled B72.3 to bind to the malignancy in the patient.

Publications

Colcher D, Esteban JM, Carrasquillo JA, Sugarbaker P, Reynolds JC, Bryant G, Larson SM, and Schlom J: Quantitative analyses of selective radiolabeled monoclonal antibody localization in metastatic lesions of colorectal cancer patients. Cancer Res. 47: 1185-1189, 1987.

Greiner JW, Horan Hand P, Colcher D, Weeks M, Thor A, Noguchi P, Pestka S, and Schlom J: Modulation of human tumor antigen expression. J. Lab. Clin. Med. 109: 244-261, 1987.

Esteban JM, Colcher D, Sugarbaker P, Carrasquillo JA, Bryant G, Thor A, Reynolds JC, Larson SM, and Schlom J: Quantitative and qualitative aspects of radiolocalization in colon cancer patients of intravenously administered MAb B72.3. Int. J. Cancer 39: 50-59, 1987.

Horan Hand P, Weeks MO, Colcher D, Thor A, Ohuchi N, Greiner J, Muraro R, Wunderlich D, Vilasi V, Petska S, Noguchi P, Cunningham R, and Schlom J,: Colon carcinoma cell populations as defined by monoclonal antibodies. In Moyer MP, and Poste GH (Eds.): Colon Cancer Cells. New York, Academic Press In Press).

Esteban JM, Schlom J, Mornex F, and Colcher D: Radioimmunotherapy of athymic mice bearing human colon carcinomas with monoclonal antibody B72.3: Histologic and autoradiographic study of effects on tumors and normal organs. European Journal of Cancer 23: 643-655, 1987.

Lan MS, Bast RC, Colnaghi MI, Knapp RC, Colcher D, Schlom J, and Metzgar, RS, Co-expression of human cancer-associated epitopes on mucin molecules. Int. J. Cancer 39: 68-72, 1987.

Carrasquillo JA, Sugarbaker P, Colcher D, Reynolds JC, Esteban J, Bryant G, Keenan AM, Perentesis P, Schlom J, and Larson SM: Radioimmunoscintigraphy of colon cancer with 131 I labeled B72.3 monoclonal antibody. J. Nuc. Med. In Press).

Ohuchi N, Wunderlich D, Fujita J, Colcher D, Muraro R, Nose M, and Schlom, J: Differential expression of carcinoembryonic antigen (CEA) in early gastric adenocarcinomas versus benign gastric lesions defined by monoclonal antibodies reactive with restricted CEA epitopes. Cancer Res. 47: 3565-3571, 1987.

Colcher D, Carrasquillo JA, Esteban JM, Sugarbaker P, Reynolds JC, Siler K, Bryant G, Larson SM, and Schlom J: Radiolabeled monoclonal antibody B72.3 localization in metastatic lesions of colorectal cancer patients. Int. J. Rad. Appl. Instrum., Part B; Nucl. Med. Biol. 14: 251-262, 1987.

Ohuchi N, Simpson JF, Colcher D, and Schlom J: Complementation of anti-CEA and anti-TAG-72 monoclonal antibodies in reactivity to human gastric adenocarcinomas. Int. J. of Cancer 40: 726-733, 1987.

Colcher D, Esteban J, Carrasquillo JA, Sugarbaker P, Reynolds JC, Bryant G, Larson SM, and Schlom J: Complementation of intracavitary and and intravenous administration of a monoclonal antibody (B72.3) in patients with carcinoma. Cancer Res. 47: 4218-4224, 1987.

Colcher D, Simpson J, Ohuchi N, Carrasquillo JA, Reynolds JC, Larson SM, and Schlom J: Potential use of monoclonal antibody B72.3 for the diagnosis and managment of gastrointestinal malignancies. In K. Sugahara (Ed.): New Trends of Gastroenterology 1987. Kyoto, Shinkoshuppan, 1987, pp. 152-165.

Carrasquillo JA, Sugarbaker P, Colcher D, Reynolds JC, Esteban J J, Bryant G, Perentesis P, Yokoyama K, Rotman M, Schlom J, and Larson SM: Peritoneal carcinomatosis: Imaging with intraperitoneal injection of I-131-labeled. B72.3 monoclonal antibody. Radiology 167: 35-40, 1988 35-40, 1988.

Schlom J, Johnston WW, Szpak CA, Colcher D, Siler K, Hand P, Sugarbaker P, Carrasquillo J, Reynolds J, and Larson SM: The use of MAb B72.3 in the diagnosis of human carcinoma. J. of Tumor Marker Oncology 2: 95-112, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09008-07 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Localization and Therapy Using Labeled Monoclonal Antibodies: Model Systems

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

David Colcher	Supv. Microbiologist	LTIB, DCBD, NCI
Mario Roselli	Visiting Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI
Patrizia Ferrone	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

O. Gansow, A. Raubitschek, Radiation Oncology, DCT, NCI
J. Carrasquillo, Department of Nuclear Medicine, CC, NIH

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	1.1	1.3

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.)*

Monoclonal antibody (MAb) B72.3 binds to many human epithelial malignancies including breast, colon, ovarian and lung cancer. Radioiodinated B72.3 IgG can selectively bind to human colorectal carcinomas grown in athymic mice; the 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. B72.3 IgG was labeled with In-111 using four chelating agents: 1-(p-isothiocyanato-benzyl)-DTPA (SCN-Bz-DTPA), MA-DTPA, CA-DTPA, and the SCN-Bz derivative of EDTA. Comparative biodistribution studies demonstrated that tumor uptake of radiolabel was very similar between the chelates (30% ID/g), however, a progressive accumulation of activity in the abdominal organs, predominantly in the liver, was seen with the MA-DTPA, CA-DTPA and SCN-Bz-EDTA chelate-antibody complexes. This uptake was very prominent with these chelate-MAb complexes but was virtually absent in the mice injected with B72.3SCN-Bz-DTPA.

The high degree of selective binding of this MAb has led to the investigation of its potential as a radioimmunotherapeutic agent. Athymic mice bearing human colon carcinoma xenografts were injected with B72.3 IgG to assess the effect of the radiolabeled MAb on the tumor growth as well as potential toxic side effects in vital organs. In mice treated with the I-131 B72.3 IgG, a marked inhibition of the growth of the human colon carcinoma xenografts was noted. Toxicity was readily evident in the mice injected with the high-dose regimen (500 µCi), with confirmed bone marrow aplasia that proved lethal for 2 of 10 animals. Lower doses (300 µCi) resulted in a bone marrow suppression of approx. 50% of the cells, which proved to be non-lethal. The tumors in the treated mice showed extensive necrosis caused by the lethal dose of I-131-B72.3 that irreversibly damaged the cells.

GPO 914-918

Major Findings

Tumor distribution studies with Iodinated B72.3. Radiolocalization studies were performed with B72.3 using athymic mice bearing human colon carcinomas (LS-174T). 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 I-125-B72.3 IgG. The ratio of %ID/gm 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, spleen, or 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. Approximately 10-20% of the injected dose per gram reached the tumor at Day 2 post-inoculation of the radiolabeled antibody. A major difference between the B72.3 system and other monoclonal antibodies 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 rising tumor:tissue ratios resulted 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 19. Studies were then undertaken to determine whether localization of the I-125 labeled B72.3 was sufficient to detect by gamma camera scanning. Athymic mice bearing colon carcinomas were given injections of approximately 70 μ Ci of I-125-B72.3 IgG. 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. With time the activity in the area of the heart and lungs significantly decreased and the proportion of the activity found in the tumor continued to increase. The background activity cleared from the mice with a T1/2 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 I-125-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.

Tumor distribution studies with In-111-labeled B72.3

Athymic mice bearing LS-174T human colon carcinoma xenografts were injected via tail vein with In-111-B72.3-SCN-Bz-DTPA. The uptake by the tumor rose 4-fold over the first two days reaching 30%ID/g at 72 h. The blood uptake dropped over the same period while the rest of the organs remained relatively constant with liver uptake values in the range of 9 to 6%ID/g from 8 to 72 hrs. Tumor to organ ratios rose uniformly over 72 h time period and consequently the images taken over that period became progressively clearer with most of the activity present in the tumor and decreasing abdominal activity over time.

We compared the above results with those obtained in parallel studies performed with three different MAb-chelates; CA-DTPA and MA-DTPA, two of the more popular bifunctional chelates and SCN-Bz-EDTA. Accumulation of the MAb-chelate conjugate in the tumor similar to that observed with In-111-B72.3-SCN-Bz-DTPA was noted with the In-111-B72.3-MA-DTPA and less deposition was seen with the In-111-B72.3-CA-DTPA. However, the percent ID per gram found in all the normal organs and particularly the liver was much higher with these chelates.

Consequently, the tumor-to-organ ratios never rose to the values obtained by In-111-B72.3-SCN-Bz-DTPA. In-111-B72.3-SCN-Bz-EDTA also proved to be an unstable preparation with tumor-to-liver ratio rising for the first 24 h, and dropping thereafter. The In-111 apparently leaked out of thermodynamically less stable chelate and accumulated in the liver, impairing the tumor images.

Radioimmunotherapy with I-131-labeled B72.3

Previous studies have demonstrated that radioiodinated B72.3 can selectively bind to human colorectal carcinomas grown in athymic mice. The high degree of selective binding of this MAb has led to the investigation of its potential as a radioimmunotherapeutic agent. Athymic mice bearing human colon carcinoma xenografts were injected with I-131-B72.3 IgG to assess the effect of the radiolabeled MAb on the tumor growth as well as potential toxic side effects in vital organs. In mice treated with the I-131-B72.3 IgG, a marked inhibition of the growth of the human colon carcinoma xenografts was noticed in comparison with control mice injected with PBS or control mice that received unlabeled B72.3 IgG. The tumors from these control mice weighed 2.7 to 3.7 times more than the tumors from the treated mice at 17 days post-inoculation of the radiolabeled MAb. Autoradiographic studies demonstrated a heterogeneous distribution of radioactivity throughout the tumor mass at 11 days post-administration of MAb. Toxicity was readily evident in the mice injected with the high-dose regimen (500 μCi), with confirmed bone marrow aplasia that proved lethal for 2 of 10 animals. The lower dose (300 μCi) resulted in a bone marrow suppression of approx. 50% of the cells, which proved to be non-lethal. The tumors in the treated mice showed extensive necrosis caused by the lethal dose of 131I-B72.3 that irreversibly damaged the cells. Radiation-induced terminal differentiation of cells was also found as manifested by the drastically decreased mitotic count (0-2 vs. 12-14 per 10 high power fields seen in control tumors) in treated animals.

Publications

Lyon RC, Faustino PJ, Cohen JS, Katz A, Mornex F, Colcher D, Baglin C, Koenig S, and Hambright P: Tissue distribution and stability of metalloporphyrin MRI contrast agents. Magnetic Resonance in Medicine 4: 24--33, 1987.

Esteban JM, Schlom J, Gansow OA, Atcher RW, Brechbiel MW, Simpson DE and Colcher D: A new method for the chelation of 111 In to monoclonal antibodies: Biodistribution and imaging of athymic mice bearing human colon carcinoma xenografts. J. Nuc. Med. 28: 861-870, 1987.

Esteban JM, Schlom J, Mornex F, and Colcher D: Radioimmunotherapy of athymic mice bearing human colon carcinomas with monoclonal antibody B72.3: Histologic and autoradiographic study of effects on tumors and normal organs. European Journal of Cancer 23: 643-655, 1987.

Colcher D, Carrasquillo JA, Esteban JM, Sugarbaker P, Reynolds JC, Siler K, Bryant G, Larson SM, and Schlom J: Radiolabeled monoclonal antibody B72.3 localization in metastatic lesions of colorectal cancer patients. Int. J. Rad. Appl. Instrum., Part B; Nucl. Med. Biol 14: 251-262, 1987.

Greiner JW, Guadagni F, Noguchi P, Petska S, Colcher D, Fisher PB and Schlom J: Recombinant interferon enhances monoclonal antibody-targeting of carcinoma lesions in vivo. Science 20: 895-898, 1987.

Colcher D, Simpson J, Ohuchi N, Carrasquillo JA, Reynolds JC, Larson SM, and Schlom J: Potential use of monoclonal antibody B72.3 for the diagnosis and management of gastrointestinal malignancies. In K. Sugahara (Ed.): New Trends of Gastroenterology 1987. Kyoto, Shinkoshuppan, 1987, pp. 152-165.

Schlom J, Colcher D, Roselli M, Carrasquillo JA, Reynolds JC, Larson SM, Sugarbaker P, Tuttle SE, and Martin EW: Tumor targeting with monoclonal antibody B72.3. Int. J. Radiation and Isotopes (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09018-04 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
Jean Simpson	Medical Staff Fellow	LTIB, DCBD, NCI
Mario Roselli	Visiting Fellow	LTIB, DCBD, NCI
Alfredo Molinolo	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)* S. Larson, J. Carrasquillo, Nuc. Med. NIH/A. Raubitschek, RO, ROB, DCT, NCI/M. Lotze, W. Sindelar, SB, NCI/P. Sugarbaker, Emory Univ./E. Martin, Ohio S Uni, Dept. Surgery/ M. Salvatore, U. Naples, Nuc. Med./ M. Merino, LP, NCI

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.7	2.2	1.5

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 and several other collaborating institutions to detect and localize carcinoma lesions using radiolabeled monoclonal antibody (MAb) B72.3. Parameters that are being 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) type of carcinoma (colorectal, ovarian, lung, breast) (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 development of human anti-murine Ig antibodies; (h) metabolism of MAb. 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).

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

Clinical trials have been initiated in collaboration with the Surgery Branch (DCT, NCI), Radiation Oncology Branch (DCT, NCI), Laboratory of Pathology (NCI), and Nuclear Medicine Dept. (NIH) at the NIH Clinical Center to define the ability of radiolabeled MAb B72.3 to localize carcinoma lesions in patients. Approximately 5-8 days prior to surgery, patients are administered radiolabeled MAb B72.3 IgG and localization of radiolabeled MAb is monitored by sequential gamma scans. At surgery, tumor and normal tissue (removed for staging purposes) biopsies are analyzed for cpm of radiolabeled MAb/gram of tissue; from these data quantitative tumor to normal tissue ratios (radiolocalization indices) are calculated. Also analyzed in all studies are: (a) serum antigen levels, (b) formation of antigen - MAb complexes, (c) pharmacokinetics of MAb serum clearances, (d) presence of development of human anti-mouse antibodies, (e) immunoperoxidase analyses of biopsied specimens for antigen expression. Several trials involving colorectal, ovarian, breast and lung carcinoma patients are beginning or are in progress at the NIH Clinical Center and several other institutions.

Parameters being investigated include: (a) effect of MAb dose and specific activity, (b) route of MAb administration: I.V., I.P., S.C., (c) choice of radionuclide: 131-I, 99m - Tc, 111-In, 125-I, (d) size, location, and other inherent properties of the tumor mass, such as antigen content. Thus far, using 131-I labeled B72.3 administered intraveneously in patients with colorectal cancer, MAb localization (tumor: normal tissue ratios of $\geq$ 3:1) was observed in approximately 75% of patients. In studies to localize carcinoma lesions in the peritoneal cavity, I.P. administered 131-I MAb B72.3 was shown to bind peritoneal implants more efficiently than I.V. co-administered MAb. Studies involving the co-administration of control MAb demonstrated the specificity of the MAb B72.3 localization.

Publications

Esteban JM, Colcher D, Sugarbaker P, Carrasquillo JA, Bryant G, Thor A, Reynolds JC, Larson SM, and Schlom J.: Quantitative and qualitative aspects of radiolocalization in colon cancer patients of intraveneously administered MAb B72.3. Int. J. Cancer 39: 50-59, 1987.

Colcher D, Esteban JM, Carrasquillo JA, Sugarbaker P, Reynolds JC, Bryant G, Larson SM, and Schlom J. Quantitative analyses of selective radiolabeled monoclonal antibody localization in metastatic lesions of colorectal cancer patients. Cancer Research 47: 1185-1189, 1987.

Colcher D, Esteban J, Carrasquillo JA, Sugarbaker P, Reynolds, JC, Bryant G, Larson SM, and Schlom J: Complementation of intracavitary and intravenous administration of a monoclonal antibody (B72.3) in patients with carcinoma. Cancer Research. 47: 4218-4224, 1987.

Sickle-Santanello BJ, O'Dwyer PJ, Mojzisik C, Tuttle SE, Hinkle, GH, Rousseau M, Schlom J, Colcher D, Thurston MO, Nieroda C, Sardi A, Minton JP, Martin EW: Radioimmunoguided surgery using the monoclonal antibody B72.3 in colorectal tumors. Diseases of the Colon & Rectum. 30: 761-764, 1987.

Colcher D, Simpson J, Ohuchi N, Carrasquillo JA, Reynolds JC, Larson SM, and Schlom, J: Potential use of monoclonal antibody B72.3 for the diagnosis and management of gastrointestinal malignancies. In: K. Sugahara (Ed.) New Trends of Gastroenterology 1987 pp. 153-165.

Renda A, Salvatore M, Sava M, Landi R, Lastoria S, Coppola L, Schlom J, Colcher D, and Zannini G.: Immunoscintigraphy in the follow-up of patients operated on for carcinoma of the sigmoid and rectum. Diseases of the Colon and Rectum. 30: 683-686, 1987.

Carrasquillo JA, Sugarbaker P, Colcher D, Reynolds JC, Esteban J, Bryant G, Perentesis P, Yokoyama K, Rotman M, Schlom J, and Larson SM: Imaging of peritoneal carcinomatosis with intraperitoneal injection. Radiology 167: 35-40, 1988.

Whittle N, Adair J, Lloyd C, Jenkins L, Schlom J, Raubitschek A, Colcher D, and Bodmer M: Expression in COS cells of a mouse-human chimaeric B72.3 antibody: Protein Engineering. Vol. 1 no. 6 pp. 499-505, 1987.

Tuttle SE, Jewell SD, Mojzisik C, Hinkle GH, Colcher D, Schlom J, and Martin EW. Intraoperative radioimmunolocalization of colorectal carcinoma using a hand-held gamma probe and MAb B72.3: Comparison of in vivo gamma probe counts with in vitro MAb radiolocalization. Int J Cancer (in press)

Schlom J, Colcher D, Roselli M, Carrasquillo JA, Reynolds JC, Larson SM, Sugarbaker P, Tuttle SE, and Martin EW. Tumor targeting with monoclonal antibody B72.3. Int J Radiation and Isotopes (in press)

Schlom J, Greiner JW, Colcher D, Larson SM, Carrasquillo JA, Reynolds JC, Sugarbaker PH, Siler K. Concepts in the delivery of monoclonal antibodies in the targeting of human carcinomas. Advanced Drug Delivery Reviews. (in press)

Schlom J, Johnston WW, Szpak CA, Sugarbaker P, Colcher D, Siler K, Thor A, Bryant G, Carrasquillo JA, Reynolds JC, Keenan AM, and Larson SM. Tumor targeting with monoclonal antibody B72.3: Experimental and clinical results, In D. M. Goldenberg (Ed.) Cancer Imaging with Radiolabeled Antibodies Martinus Nijhoff Publishing, Norwel, MA. (in press)

Martin EW, Mojzisik CM, Hinkle GH, Sampsel J, Siddiqi MA, Tuttle SE, Sickle-Santanello B, Colcher D, Thurston MO, Bell JG, Farrar WB, and Schlom J. Radioimmunoguided surgery: A new approach to the intraoperative detection of tumor using monoclonal antibody B72.3. Amer J Surgery (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09012-04 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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)*

Jean Simpson	Medical Staff Fellow	LTIB, DCBD, NCI
Alfredo Molinolo	Visiting Fellow	LTIB, DCBD, NCI
Kai Chang	Visiting Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Drs. W. Johnston and C. Szpak, Dept. of Path., Duke U. Durham, NC
Dr. F. Gorstein, Department of Pathology, Vanderbilt Univ., Nashville, TN
Dr. N. Ohuchi, Department of Surgery, Sendai City Hospital, Sendai, Japan

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

A

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

Monoclonal antibodies (MAbs) have been utilized with immunohistochemical methods for the (a) detection of occult carcinoma in surgical and cytology preparations, (b) phenotyping of malignant cell populations, (c) differentiation of histologic tumor types, and (d) identification of various cellular products. MAbs with selective reactivity against tumor associated antigens have specifically been adapted for use with cytologic preparations including cytospins, membranes, and fine needle aspiration biopsies (FNAB) for the detection and differentiation of carcinomas from benign cell types and other cancerous lesions.

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

Major Findings

A monoclonal antibody 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, (termed TAG-72) a novel high molecular weight glycoprotein complex, can be detected in formalin-fixed, paraffin-embedded tissue sections of human breast, colon, and ovarian carcinomas, and not in a variety of normal adult human tissues. We have also determined that B72.3 may be used as an adjunct for diagnosis of carcinoma in cytologic preparations (cell pellets and cytospins) of human effusions as well as in FNAB specimens. Using the avidin-biotin complex method of immunoperoxidase staining, B72.3 was used to identify carcinoma cells in effusions from various organs including: lung (non-small cell carcinoma) (36/40), breast (46/53), ovary (110/113), gastrointestinal tract (14/17), and carcinomas from unknown primary sites (37/48). Benign effusions (n=821) were negative. B72.3 was also used to identify carcinoma cells in fine needle aspiration biopsies from the lung (non-small cell carcinoma) (145/157), breast (40/42), ovary (10/11), gastrointestinal tract (36/41), and prostate (13/19). Benign lesions of the lung, breast, pancreas, and lymph nodes were generally non-reactive. In addition, B72.3 was non-reactive with FNABs of small-cell carcinoma of the lung, melanoma, sarcoma, and lymphoma. Thus, these data suggest that the immunocytological application of MAb B72.3 should now be considered as an adjunct in the discrimination of carcinoma cells from benign cells both in effusions and FNABs. In addition, B72.3, may be of use in the discrimination of tumor types, particularly adenocarcinoma vs mesothelioma, small cell carcinoma, melanoma, or lymphoma.

Further analysis of the distribution of TAG-72 in benign and malignant tissues has been completed. B72.3 was found to react with 100% of secretory endometria (n=38) whereas proliferative endometria (n=7) and resting endometria (n=10) were uniformly negative. In addition, 100% of endometrial carcinomas (n-32) were reactive with B72.3.

MAbs with selective CEA reactivity (termed COL MAbs) have also been developed. These MAbs are currently being evaluated for their reactivites to human tissues using immunoperoxidase techniques for potential clinical, immunopathological, and diagnostic applications. MAbs COL-1,4,6, and 12 were used to study the differential expression of CEA in benign and malignant gastic tissues. Each of the COL-MAbs showed selective reactivity with adenocarcinoma vs benign or normal gastric tissues, and were able to detect early carcinomas (confined to the mucosa) in 7/10 cases, with COL-4 detecting 10/10 early carcinomas.

In order to detect greater numbers of carcinoma cells the use of a combination of B72.3 and COL-4 was used to study 17 gastric carcinomas. By a double-staining technique using avidin-biotin peroxidase and avidin-biotin-alkaline phosphatase, 13/17 cases showed greater than 90% of cells reactive with one or both MAbs, whereas B72.3 used singly detected 90% of carcinoma cells in only one case, and COL-4 detected 90% of carcinoma cells in 2 cases.

Publications

Thor A, Viglione M, Muraro R, Ohuchi N, Schlom J, and Gorstein F: Monoclonal antibody B72.3 reactivity with human endometrium: a study of normal and malignant tissues. Int. J. Gyn. Path 6: 235-247, 1987.

Thor A, Simpson J, Ohuchi N, Horan Hand P, Szpak C, Johnston W, and Schlom J: Monoclonal antibodies and human carcinomas: diagnosis and experimental applications. In: Advances in Immunohistochemistry, ed. R. DeLellis (In press)

Johnston W, Szpak C, Thor A, Simpson J, and Schlom J: Applications of immunocytochemistry to clinical cytology. Cancer Inv 5 (6), 593-611 (1987)

Ohuchi N, Wunderlich D, Fujita J, Colcher D, Muraro R, Nose M, and Schlom J: Differential expression of carcinoembryonic antigen in early gastric adenocarcinomas versus benign gstric lesions defined by monoclonal antibodies reactive with restricted antigen epitopes. Cancer Research 47: 3565-71, 1987.

Ohuchi N, Simpson J, Colcher D, and Schlom J: Complementation of anti-CEA and anti-TAG-72 monoclonal antibodies in reactivity to human gastric adenocarcinomas. Int. J. Cancer. 40: 726-733, 1987.

Johnston W W, Szpak C, Thor A, Simpson J, and Schlom J: Antibodies to tumor-associated antigens: Applications in clinical cytology. In Wied, Koss, and Reagan (Ed.): 5th Edition of Compendium on Diagnostic Cytology, International Academy of Cytology. pp. 567-578.

Thor A, Viglione M J, Ohuchi N, Simpson JF, Steis R, Cousar J, Lippman M, Kufe DW, Schlom J: Comparison of monoclonal antibodies for the detection of occult breast carcinoma metastases in bone marrow. Breast Cancer Res. Treat. (In press)

Simpson J, and Schlom J: The use of monoclonal antibodies in the managment of gynecologic malignancies: The Yale Journal of Biology and Medicine (In press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09021-02 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Molecular Cloning and Analysis of Tumor-Associated Antigens

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

Judy Kantor	Expert	LTIB, DCBD, NCI
Paul Robbins	Staff Fellow	LTIB, DCBD, NCI
Scott Meissner	Staff Fellow	LTIB, DCBD, NCI
Shannon Dixon	Microbiologist	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

G. Shivley, City of Hope Hospital, Duarte, California

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Studies carried out in our lab as well as others have demonstrated the presence of the TAG-72 and CEA tumor associated antigens in a high percentage of human carcinomas. Our studies have focused on understanding the role of these molecules in tumorigenesis as well as understanding the molecular mechanisms involved in the regulation of expression of these antigens. Specific probes for members of the CEA gene family will be used to study their expression in various tissues and cell lines and to examine the mechanism of regulation of CEA expression by interferons as well as other biological response modifiers. In addition, the gene encoding CEA will be cloned and transfected into mouse metastatic cell lines in order to obtain a mouse model for the study of anti-tumor agents.

Polyclonal antisera to deglycosylated TAG-72 and monoclonal antibodies to native TAG-72 will be used to obtain clones from cDNA libraries. Positive clones will be sequenced and used for analysis of TAG-72 expression in normal and tumor cell lines and tissues.

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

Major Findings

CEA project: The expression of CEA, as determined by MAb binding, was found to be modulated by interferon in certain colon carcinoma cell lines. The initial studies indicated a correlation between protein expression and mRNA levels in these cell lines. Further studies of methylation status, chromatin structure, and transcriptional control in these cell lines will be carried out to determine their role in expression of CEA in these cell lines.

TAG-72 project: Polyclonal antisera raised against the deglycosylated antigen have been used to screen bacteriophage expression libraries prepared from those breast and colon tumor cell lines known to express the highest levels of antigen. Several clones have been isolated, and DNA sequence analysis of them has demonstrated that they share limited similarity with other genes encoding mucin-like cellular and carcinoma-associated antigens. These cDNAs are being used as probes in Northern blot analyses to determine whether the expression of these genes correlates with the presence of the TAG-72 antigen. These studies should assist in the identification of the gene(s) that encode the protein component of this antigen.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08226-12 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
K. Nicholas	Guest Worker	LTIB, DCBD, NCI
E. Ginsburg	Biologist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. Sandra Haslam, Michigan State University, East Lansing, MI
Dr. Randy Whitcomb, Developmental Endocrinology, NICHD
Dr. Susan Bates, Pediatrics Branch, NCI

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

This project is designed to determine the role of hormones and growth factors in the development and differentiation of normal mammary gland and in the development, growth, and maintenance of mammary tumors. Studies include: 1) examining the roles of epidermal growth factor and mammary gland-derived growth factors in lobuloalveolar 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) examining the effect of sialadenectomy on mammary tumor incidence and growth in mice as well as the production of preneoplastic hyperplastic alveolar nodules; and 4) defining the hormone receptor and response status of the NOG-8 mouse mammary epithelial cell line.

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

Major Findings

Sialadenectomy (Sx) results in temporary growth retardation of the spontaneous mouse mammary tumor. After a period of 5-7 days, growth resumes at a rate similar to that of tumors in sham-operated animals. Acid alcohol extracts of tumors from Sx and control mice showed that they contained equivalent amounts of EGF receptor competition activity per milligram of protein. Neither extract contained α-TGF detectable by RIA. Tumors from both control and Sx animals had equal amounts of growth factor receptors.

Similar extracts of "normal" reduction mammoplasty human breast that was rapidly proliferating at the time of surgery contained low levels of EGF and RIA-detectable α-TGF at a level comparable to those found in breast neoplasms. By soft agar growth assay, the patient's serum was found to contain both α- and β-TGFs at concentrations higher than in control serum. RIA shows EGF in the serum at the same level as in control serum. The human breast tissue contains significant levels of growth factor receptor that recognizes both EGF and α-TGF. Primary cultures of the human breast epithelium secreted α-TGF into the medium at a level comparable to that of the human MCF-7 breast cancer cell line. Examination of the growth factor receptors in the human tissue showed the same affinity of both EGF and α-TGF for EGF receptor, while α-TGF receptors have a higher affinity for α-TGF than EGF. This pattern is distinct from that of either the MCF-7 cells or normal mouse mammary glands. We also found that α-TGF is more effective than EGF in promoting lobuloalveolar development of estrogen and progesterone-primed mouse mammary glands, both in vivo and in vitro.

We have developed an assay that allows us to determine both EGF and prolactin receptors on the same membrane sample. Preliminary data using growing and regressing DMBA rat mammary tumors shows an inverse relationship between the level of prolactin receptors and EGF receptors in these membranes.

NOG-8 cells have been found to contain receptors for insulin, glucocorticoids, prolactin, and thyroid hormones. The receptor content is cell density dependent. Growth of the cells is enhanced by the presence of prolactin and thyroid hormones. These cells will be used to study prolactin-thyroid hormone interactions in the tumorigenic process by introducing various genes (growth factor and oncogenes) regulated by a hormonally (prolactin) responsive promoter.

Publications

Rothschild TC, Boylan ES, Calhoon RE, Vonderhaar BK. Transplacental effects of diethylstilbestrol on mammary development and tumorigenesis in female ACI rats. Cancer Res 1987;47:4508-16.

Vonderhaar BK. Local effects of EGF, α TGF and EGF-like growth factors on lobulo-alveolar development of the mouse mammary gland in vivo. J Cell Physiol 1987; 132:581-4.

Bhattacharjee M, Weintroub S, Vonderhaar BK. Milk protein synthesis by mammary glands of vitamin D-deficient mice. Endocrinology 1987;121:865-74.

Vonderhaar BK. Regulation of development of the normal mammary gland by hormones and growth factors. In: Lippman ME, Dickson R, eds. Breast cancer: cellular and molecular biology. New York: Martinus Nijhoff, 1988;251-66.

Vonderhaar BK, Ziska SE. Hormonal regulation of milk protein gene expression. Annu Rev Physiol 1988;51:in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08274-07 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
Suzanne Ziska	Staff Fellow	LTIB, DCBD, NCI
Erika Ginsburg	Biologist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. Rhoda Maneckjee, Medical Oncology Branch, NCI, Bethesda, MD
Dr. Anthony Capuco, USDA, Beltsville, MD

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.5	0.7

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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) that affect the 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; 3) characterization of the nature of the interaction of Tamoxifen with membrane-bound receptors related to the lactogen receptor; and 4) definition of the relationship of monoclonal antibody B6.2 to human lactogenic hormone receptors.

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

Major Findings

Using an IgG fraction of a polyclonal antibody raised in rabbits against the 40-kD binding unit of the prolactin (Prl) receptor, we immunopurified Prl receptors from mouse hepatic membranes. Two major bands of approximately 90 kD and 40 kD were obtained. After preparative SDS-PAGE, the 90- and 40-kD regions of the gel were extracted and the receptor reconstituted. Only the 90-kD region retained specific Prl binding activity. Re-electrophoresis of the 90-kD form resulted in two bands of 90 and 40 kD. V-8 digestion of the 90- and 40-kD forms resulted in several common bands, suggesting that the smaller form originates from the larger form. Trypsin does not digest either form. An affinity purified receptor (using an oPrl affinity column) from human chorion-decidua membranes showed two major bands on SDS-PAGE and silver staining under both reducing and nonreducing conditions (90 kD and approximately 40 kD).

The treatment of the 90-kD unit with endoglycosidase F, an enzyme that cleaves complex high mannose-containing sugars, resulted in a 68-kD form. Treatment of membrane-bound receptors with endoglycosidase F resulted in loss of Prl binding activity. We continued to examine the relationship of monoclonal antibody B6.2 to the Prl receptor. Using an immunoaffinity column of B6.2, the membrane-bound antigens were isolated from LS174T human colon carcinoma grown in nude mice. On SDS-PAGE, three bands were seen with silver staining. Two major bands were at 90 and 40-50 kD. A minor band at about 68 kD was also seen. Extraction of the major bands from the gels run on LS174T isolates, followed by renaturation of the proteins, resulted in specific Prl binding in the 90-kD region.

We have begun to define the relationship of membrane-bound antiestrogen binding sites (AEBS) to Prl receptors. We have found that Prl receptors purified from MCF-7 tumors by oPrl affinity column retained a low level of antiestrogen activity, suggesting that the AEBS and the Prl receptor co-purify. Antiestrogens block Prl-induced growth of estrogen nonresponsive Nb2 rat lymphoma cells and MCF-7 human breast cancer cells. The ability of the antiestrogen to block growth parallels its ability to bind to the AEBS.

Publications

Vonderhaar BK, Biswas R. Prolactin effects and regulation of its receptors in human mammary tumor cells. In: Medina D, Kidwell WR, Heppner G, Anderson E, eds. Cellular and molecular biology of experimental mammary cancer. New York: New York: Plenum Publishing Co., 1987;205-19.

Vonderhaar BK. Prolactin: transport, function and receptors in mammary gland development and differentiation. In: Daniel CW, Neville MC, eds. The mammary gland: development, regulation and function. New York, Plenum Publishing Co., 1987;383-438.

Biswas R, Vonderhaar BK. Role of serum in prolactin responsiveness of MCF-7 human breast cancer cells in long term tissue culture. Cancer Res 1987;47:3509-14.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09024-01

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Parameters Involved in the Tumor Targeting of Monoclonal Antibodies

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

Patricia Horan Hand	Chemist	LTIB, DCBD, NCI
Shashi Shrivastav	Visiting Fellow	LTIB, DCBD, NCI
Jean Simpson	Medical Staff Fellow	LTIB, DCBD, NCI
Alfredo Molinolo	Visiting Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. Andrew Raubitschek, Radiation Oncology Branch, National Cancer Institute
Dr. Philip Snoy, Center for Drugs and Biologics, Division of Product Quality Control, NIH

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	1.2	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.)*

One of the problems observed in studies using radiolabeled MAbs for detection of human tumors is the relatively low percent of the injected dose of monoclonal antibody (MAb) per gram of tumor. Recent studies in in vivo model systems for melanoma and hepatoma, and clinical trials involving hepatoma patients have suggested enhanced deposition of radiolabeled antibody in tumors pretreated with external irradiation. One major goal of this project is to identify parameters that optimize binding of MAbs to carcinoma cells in vivo. We have recently initiated studies to determine the effect of exposure of carcinomas to external irradiation prior to localization of tumors by MAb conjugates. These studies include evaluation of the effect of external irradiation on (a) growth of carcinoma xenografts, (b) cellular integrity and the expression of TAG-72 at the cellular level, and, (c) binding of MAb B72.3 to carcinoma xenografts.

One approach to the treatment of patients with peritoneal carcinoma may be direct infusion into the peritoneal cavity of radiolabeled antibody conjugates. In comparison to intravenous MAb administration, this method may improve the percent of the injected MAb dose per gram of tumor and may lead to less radiolabeled antibody in the bone marrow and, therefore, less toxicity to the patient. The second major goal of this project is to develop an in vivo model system to study immunotherapy of human carcinoma in the peritoneal cavity. These studies will include (a) determination of the animal species eliciting a pattern of clearance of MAb from the blood most comparable to that observed in the human, (b) development of a human peritoneal carcinoma xenograft in an animal model, and (c) determination of the utility of intraperitoneally administered MAb conjugates for therapy of carcinoma.

PHS 6040 (Rev. 1/84)

GPO 914-919

Major Findings

The project on the identification of parameters that affect binding of MAbs to tumor cells in vivo has led to studies of the effect of irradiation on MAb targeting of human carcinomas. As a model system we employed MAb B72.3 and the LS-174T human colon carcinoma xenograft in athymic mice. LS-174T tumors exposed to 300 cGy grew to approximately 93% the size of non-irradiated tumors, while those exposed to doses of 600 - 2,000 cGy were approximately 41% the size of control tumors. Histochemical evaluation of the carcinomas revealed cellular changes consistent with early effects of radiation exposure. Expression of Tumor Associated Glycoprotein (TAG)-72, the high molecular weight mucin detected by MAb B72.3, was not affected by tumor irradiation. Exposure of carcinomas to 300 or 900 cGy in a single fraction or 900 cGy split in three-300 cGy fractions did not yield a consistent or substantial enhanced localization of radiolabeled MAb B72.3 IgG or F(ab)'2 to tumors. Only minor augmentation of MAb binding to tumors was observed in pre-irradiated mice. These results demonstrate that pre-irradiation of carcinoma does not necessarily lead to better MAb deposition in tumor and thus one should re-evaluate the use of pre-irradiation of tumor in clinical trials toward this end.

Studies are now in progress to develop an in vivo model system for immunotherapy of human carcinoma in the peritoneal cavity. Clearance of MAb B72.3 administered i.v. and i.p. will be evaluated in different strains of mice and rats, and in different species of monkeys. The in vivo model system that most closely mimics clearance of MAb B72.3 in humans will be chosen for development and optimization of therapeutic regimens using MAbs.

Publications:

Colcher D, Esteban J, Carrasquillo JA, Sugarbaker P, Reynolds JC, Bryant G, Larson SM, Schlom, J. Complementation of intracavitary and intravenous administration of a monoclonal antibody (B72.3) in patients with carcinoma, Cancer Research 1987;47:4218-4224.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09025-01

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Heterobifunctional Antibodies in Tumor Targeting

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

F. James Primus	Cancer Expert	LTIB, DCBD, NCI
Tribhuvan K. Pendurthi	Guest Researcher	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. David Segal, Immunology Branch, DCBD, NCI, NIH

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

A

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

The anti-tumor activity of T-cells, NK cells, and macrophages can be augmented by lymphokines and cytokines, monoclonal antibodies (Mabs) against tumor antigens, and combinations of Mabs and effector cell activators. Heteroantibodies with dual specificity were constructed by chemically cross-linking Mab against the human T-cell CD3 antigen with antibodies recognizing surface antigens on colon and ovarian tumors. Utilizing normal human peripheral blood lymphocytes, it was shown that these heteroantibodies, and in some cases, monomeric anti-tumor Mab, potentiated the killing of cultured tumor cells. In addition, heteroantibody-directed cytotoxicity was enhanced by effector cell activation with either IL-2 or anti-CD3 Mab. Thus, heteroantibodies may provide a new immunotherapeutic strategy for targeting cytotoxic immune cells to tumors.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings

A murine IgG1 monoclonal antibody (Mab) prepared against breast cancer could be cross-linked to the IgG2a anti-CD3 Mab via a disulphide linkage. Heteroantibody preparations containing predominantly dimers or a mixture of larger molecular weight polymers retained full immunoreactivity with lymphocytes or tumor cells, comparable to that of the respective monomeric Mabs. Immunoadsorbants containing isotype-specific antibodies showed that the heteroantibodies had bifunctional specificity. When normal human peripheral blood lymphocytes were tested for their ability to kill in vitro human colon tumor cell lines or an ovarian ascites tumor carried in nude mice, it was found that the heteroantibody potentiated cytotoxicity. This augmentation was observed more often following pre-activation of effector cells with IL-2. Dimeric and larger sized heteroantibodies were similar in their ability to mediate target cell killing. However, with some donors the killing obtained with heteroantibodies did not exceed that achieved with monomeric Mab or activated effector cells alone. Activation of NK-cell depleted lymphocytes with anti-CD3 in the presence of monocytes provided an effector cell population, which in the presence of heteroantibody but not monomeric anti-tumor Mab, resulted in augmentation of tumor cell lysis. These studies indicate that heteroantibodies in combination with effector cell activators can enhance tumor cell lysis in a specific fashion.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09026-01

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Characterization of Human Tumor-Associated Antigens

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

Phillip Fernsten	Expert	LTIB, DCBD, NCI
Donald Sheer	BPT Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

Human adenocarcinoma cells secrete and express on their cell surfaces high molecular weight, mucin-like glycoproteins that bear epitopes that are highly restricted to carcinoma cells. Monoclonal antibody B72.3, which has been extensively characterized as to reactivity with a variety of carcinomas versus normal tissues, reacts with such a mucin-like glycoprotein. The B72.3-reactive antigen, designated TAG (tumor-associated glycoprotein) -72, was partially purified and used as immunogen to produce second generation anti-TAG-72 antibodies. One of these second generation antibodies was selected to develop a procedure to purify preparative amounts of TAG-72. TAG-72, extracted from xenografted human colon carcinoma cells, was subjected to antibody affinity chromatography, size exclusion chromatography, and ion exchange chromatography. A double determinant radioimmunoassay revealed a greater than 1000-fold purification with a greater than 10% yield. SDS-PAGE analysis of radiolabeled purified TAG-72 revealed a polydisperse, but apparently homogenous, high molecular weight glycoprotein. Amino acid analysis of the purified TAG-72 revealed a profile highly similar to that reported for other purified mucins. The purified TAG-72 has been partially deglycosylated with trifluoromethane sulfonic acid in preparation for amino acid sequence analysis.

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

Major findings

Xenografts of the LS174T human colon carcinoma cell line have been shown in a double determinant radioimmunoassay to contain levels of the B72.3-reactive antigen, TAG-72, equivalent to the highest levels found in primary and metastatic human colon and breast carcinomas. Western blotting analyses of LS174T extracts revealed that the antigen had a molecular size distribution highly similar to that of TAG-72 derived from human primary and metastatic breast and colon carcinomas. Due to its availability, LS174T xenograft was therefore chosen as starting material for the purification of preparative amounts of TAG-72. CC49, a second generation antibody elicited by partially purified TAG-72, was selected for utilization in a larger scale purification scheme because of its higher affinity.

Human adenocarcinoma cells secrete and express on their cell surfaces high molecular weight, mucin-like glycoproteins that bear epitopes that are highly restricted to carcinoma cells. Monoclonal antibody B72.3, which has been extensively characterized as to reactivity with a variety of carcinomas versus normal tissues, reacts with such a mucin-like glycoprotein. The B72.3-reactive antigen, designated TAG (tumor-associated glycoprotein) -72, was partially purified and used as immunogen to produce second generation anti-TAG-72 antibodies. One of these second generation antibodies was selected to develop a procedure to purify preparative amounts of TAG-72. TAG-72, extracted from xenografted human colon carcinoma cells, was subjected to antibody affinity chromatography, size exclusion chromatography, and ion exchange chromatography. A double determinant radioimmunoassay revealed a greater than 1000-fold purification with a greater than 10% yield. SDS-PAGE analysis of radiolabeled purified TAG-72 revealed a polydisperse, but apparently homogenous, high molecular weight glycoprotein. Amino acid analysis of the purified TAG-72 revealed a profile highly similar to that reported for other purified mucins. The purified TAG-72 has been partially deglycosylated with trifluoromethane sulfonic acid in preparation for amino acid sequence analysis.

Publications

Muraro R, Kuroki M, Wunderlich D, Poole DJ, Colcher D, Thor A and Schlom, J. Generation and characterization of second generation (B72.3) monoclonal antibodies reactive with the TAG-72 antigen. Cancer Research (in press), 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09022-02 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Cytoskeletal Proteins in Oncogenic Transformation and Human Neoplasia

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
Basudev Bhattacharya	Visiting Fellow	LTIB, DCBD, NCI
Gaddamanugu L. Prasad	Visiting Fellow	LTIB, DCDB, NCI
Rebecca Fuldner	Biotechnology Res. Fellow	LTIB, DCDB, NCI
Donald Sheer	Biotechnology Res. Fellow	LTIB, DCDB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular & Molecular Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	1.9	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.)*

Studies have continued on the role of derangements in tropomyosin (TM) expression and utilization in neoplastic transformation. Neoplastic transformation secondary to expression of retroviral oncogenes is correlated with the production of TM-deficient, structurally defective, microfilaments in both fibroblasts and epithelial cells. This may be a basic structural and functional lesion which is conducive to expression of components of the transformed phenotype. The derangement results either from suppression of synthesis and utilization of TM (fibroblasts), or from relative over-expression of actin (epithelial cells). TM suppression in fibroblasts transformed by retroviral oncogenes may be due to the action of alpha-transforming growth factor produced as a consequence of oncogene expression. TM expression was also studied in a panel of established human breast cancer lines. Consistent abnormalities in tropomyosin expression were observed in the tumor cell lines, suggesting that derangement of TM expression may be a frequent event in human mammary neoplasia.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major findings

Studies have continued on the role of derangements in tropomyosin (TM) expression and utilization in neoplastic transformation. Neoplastic transformation secondary to expression of retroviral oncogenes is correlated with the production of TM-deficient, structurally defective, microfilaments in both fibroblasts and epithelial cells. This may be a basic structural and functional lesion which is conducive to expression of components of the transformed phenotype.

In NIH-3T3 fibroblasts transformed by a variety of retroviral oncogenes, marked suppression of TM synthesis and impaired utilization of TM for microfilament formation was found. In NMuMG (normal mouse mammary gland) epithelial cells, abnormal TM:actin ratios in microfilaments were associated with elevated actin synthesis rather than TM suppression. Thus, the biochemical mechanism of impaired microfilament formation related to retroviral oncogene expression may be conditioned by the differentiated state of the cells involved.

In order to clarify the specific role of TM suppression in production of the transformed phenotype, we are constructing a vector in which expression of an antisense sequence to the initiation region of TM mRNA will be placed under glucocorticoid control. After transfection into NRK fibroblasts, we will assess:

1. The ability of glucocorticoids to induce antisense expression in the transfected cells.
2. Effects of such expression on TM production
3. Effects on cell growth characteristics, such as anchorage independent growth.
4. Tumorigenicity in nude mice.

TM suppression in fibroblasts transformed by retroviral oncogenes may be due to the action of alpha-transforming growth factor (a-TGF) known to be produced as a consequence of oncogene expression. NRK rat fibroblasts treated with a-TGF exhibited rapid suppression of synthesis and utilization of the same specific isotype of TM found to be suppressed when oncogenes are expressed.

To examine further the relationships between a-TGF, TM suppression and transformation, we are studying the fibroblast line, NR6, which lacks expression of the receptor for a-TGF (EGF receptor). Derivatives transformed by retroviral oncogenes will be produced and effects on microfilament proteins and on the expression of EGF receptor mRNA will be studied. A vector is being constructed which will permit glucorticoid regulated expression of the activated c-Ha-*ras* transforming sequence. When transfected into NR6 cells, this will permit assessment of the specific role of p21-*ras* on cell phenotype and TM metabolism in the absence of effects due to a-TGF.

TM expression was also studied in a panel of established human breast cancer lines and compared with immortalized normal, and primary mammary epithelial cells. The set of TMs expressed by mammary epithelial cells was characterized and shown to be different from those expressed by human

fibroblasts. All of the mammary tumor epithelial cell lines showed abnormalities in tropomyosin expression. Higher molecular weight TMs were consistently absent, and overexpression of a 35 kDa TM was generally found. Preliminary examination of colon and epidermal carcinoma cell lines did not show these abnormalities. These results suggest that derangement of TM expression may be a frequent event in specific types of human neoplasms, notably in breast cancer.

Work with human mammary tumor cells will be pursued by correlating expression of the various TM proteins with TM mRNAs, as detected by northern blot analysis using a number of specific probes already available and others being developed by us. We are screening a human epithelial cell cDNA library to recover clones specific for the TMs expressed in epithelial cells. By sequencing such cDNAs, we will be able to examine the relationship between the TM mRNAs expressed in fibroblasts and in epithelial cells in order to determine whether the different cell lineages express products of different, related genes, or engage in alternative splicing to produce different messages from the same set of genes. The availability of cDNA probes specific for epithelial cell TMs will permit Southern blot analysis of tumor cell DNA to screen for genetic rearrangements involving the TM loci. To facilitate these studies, a panel of monoclonal antibodies to human epithelial cell tropomyosins will be produced. Further extension of this work will include screening of fresh human tumor biopsy material for TM expression, to determine if these reflect the derangements found in long-term cell cultures.

Publications

Cooper HS, Bhattacharya B, Bassin RH, and Salomon DS: Suppression of synthesis and utilization of tropomyosin in mouse and rat fibroblasts by alpha-transforming growth factor: a pathway in oncogene action. Cancer Research, 47: 4493-4500, 1987.

Bhattacharya B, Ciardello F, Salomon DS, and Cooper HL: Disordered metabolism of microfilament proteins, tropomysin and actin, in mouse mammary epithelial cells expressing the Ha-ras oncogene. Oncogene Research, In press, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09006-06 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Studies on the Nature and Function of the Phosphoprotein, Prosolin

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

Herbert L. Cooper Chief, Cell and Molec. Phys. Sect. LTIB, DCDB, NCI

Richard Braverman Chemist LTIB, DCDB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular & Molecular Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.0	0.8

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.)*

Studies have continued on the unique 18.4K, pI 5.9 phosphoprotein, prosolin (formerly called pp17), a major cytosolic protein which undergoes rapid phosphorylation in HL60 promyelocytic leukemia cells when their growth is inhibited and they are induced to differentiate in response to treatment with phorbol ester (TPA). The expression and phosphorylation of prosolin in human peripheral lymphocytes (PBL) was investigated. Prosolin was identified in PBL, and its expression was found to be correlated with the S-phase of the cell cycle. In proliferating PBL prosolin was a major cytosolic component, comprising 0.5% of total cytosolic protein, of which 25% was found in 2 phosphorylated forms. TPA treatment of proliferating PBL caused phosphorylation of about 2/3 of pre-existing unphosphorylated prosolin within 1 hr followed immediately by an abrupt cessation of DNA synthesis. Phosphorylation of prosolin may be an initiating event in the antiproliferative response to TPA. The availability of prosolin only during S-phase may provide a regulatory mechanism by which naturally occuring cytokines may terminate lymphocyte growth, through activiation of antiproliferative processes linked to phosphorylation of prosolin, without inhibiting activation of G_0 or G_1 cells.

Major findings:

Studies have continued on the unique 18.4K, pI 5.9 phosphoprotein, prosolin (formerly called ppl7), a major cytosolic protein which undergoes rapid phosphorylation in HL60 promyelocytic leukemia cells when their growth is inhibited and they are induced to differentiate in response to treatment with phorbol ester (TPA). The expression and phosphorylation of prosolin in human peripheral lymphocytes (PBL) was investigated. Prosolin was identified by tryptic peptide mapping in PBL, and its expression was found to be correlated with the S-phase of the cell cycle. In proliferating PBL prosolin was a major cytosolic component, comprising 0.5% of total cytosolic protein, of which 25% was found in 2 phosphorylated forms. TPA treatment of proliferating PBL caused phosphorylation of about 2/3 of pre-existing unphosphorylated prosolin within 1 hr followed immediately by an abrupt cessation of DNA synthesis. Phosphorylation of prosolin may be an initiating event in the antiproliferative response to TPA. The availability of prosolin only during S-phase may provide a regulatory mechanism by which naturally occurring cytokines may terminate lymphocyte growth, through activation of antiproliferative processes linked to phosphorylation of prosolin, without inhibiting activation of G_0 or G_1 cells.

Prosolin was also found to be a prominent component of the cytosol of various rapidly dividing lymphoma and lymphatic leukemia cell lines of both B- and T-cell origin, and was expressed at more than 3 times the level of growing normal lymphocytes. The relation of this over-expression to neoplastic growth is being explored by an examination of the phosphorylation response to TPA in these cells. Preliminary data indicate that neoplastic lymphoid cells, although they over-express prosolin, are defective in their ability to phosphorylate the molecule in response to TPA, in comparison to normal PBL.

To pursue this project, partial amino acid sequencing has been performed on tryptic peptides. From these sequences, oligodeoxynucleotide probes will be made to screen cDNA libraries for cloning and sequencing of prosolin cDNA. Polyvalent antiserum to synthetic peptides based on the available amino acid sequence data is also being raised as an aid in this related projects.

To investigate the possibility that phophorylation of prosolin is involved in the response of PBL to naturally occurring growth regulatory cytokines, the ability of various materials to produce rapid phophorylation of prosolin in PBL is being assessed. These materials include prostaglandins, cyclic nucleotides, transforming and other growth factors, lymphokines, ionophores, and suppressor T-lymphocytes.

In other studies, the possible role of prosolin and its phosphorylated forms in mRNA stabilization is being pursued.

Publications

Cooper HL, and Braverman R: Phosphorylation of prosolin and leukocyte growth regulation. In: Lymphocyte Activation and Differentiation, J.C. Mani & J. Dornand, Eds. DeGruyter & Co., Berlin, 1988, pp. 67-72.

Cooper HL, McDuffie E, and Braverman R: Human peripheral lymphocyte growth regulation and response to phorbol esters is linked to synthesis and phosphorylation of the cytosolic protein, prosolin. J. Immunol. In press, 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04848-16 LTIB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
RNA Tumor Viruses: Replication, Transformation, and Inhibition in Cell Cultures

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

R. Bassin	Chief, Biochem. of Oncogenes Sect.	LTIB, DCBD, NCI
B. Blondel	Visiting Fellow	LTIB, DCBD, NCI
G. Tortora	Visiting Fellow	LTIB, DCBD, NCI
D. Salomon	Research Biologist	LTIB, DCBD, NCI
R. Callahan	Chief, Oncogenetics Section	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*
Dr. Arie Moran, Ben Gurion University of the Negev, Beer-sheva, Israel
Dr. Len Benade, American Red Cross, Rockville, MD
Dr. Donald Blair, DCCP at Frederick

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Biochemistry of Oncogenes Section

INSTITUTE AND LOCATION
NCI, Bethesda, Maryland 20892

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.)*

Our previous studies on the mechanism of transformation by retroviral oncogenes have indicated that there are fundamental differences in monovalent cation transport in NIH/3T3cells and other cell lines following transformation by the oncogene ras. These differences are "corrected" in flat revertant cells that resist transformation by ras and certain other oncogenes. Using sodium uptake and pH measurements, we have now shown that ras-transformed cells do indeed show altered sodium/hydrogen antiport activity when compared to controls. The mechanisms by which revertant cells require resistance to ouabain is under investigation. The sodium/hydrogen antiporter is known to be stimulated by transforming growth factor alpha and may play a critical role in the control of cell division.

We are continuing to isolate new cellular mutants that are resistant to transformation by retroviral oncogenes. The proline analog 4-cis-hydroxyproline is less toxic for normal cells than for transformed cells. We have demonstrated that cis-hydroxyproline can be used to isolate nontransformed (revertant) cells from populations of both ras and raf transformed cells. The properties of these revertants are now being compared with those of the initial ras revertants isolated following treatment with ouabain.

Retroviral vectors have many advantages over other DNA transfer techniques, including a stable integration of single copies of the gene of interest and introduction into a wide variety of cells with a high transfer frequency. We are now in the process of constructing new retroviral vectors containing oncogenes so that we can engineer cells useful for the isolation of a second generation of transformation resistant cells.

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

Major Findings

Our original finding that transformed cells are more sensitive to the toxicity of ouabain than are their nontransformed counterparts indicated that change(s) in monovalent cation transport might be associated with the transformed state. Two other results, (1) the isolation of nontransformed revertant cells with low sensitivities to ouabain and (2) the finding that transformed cells show a supression of synthesis of tropomyosin (shown by others to markedly increase the sensitivity of partially purified Na, K ATPase) also indicated that normal and transformed cells might differ in monovalent cation transport.

During the past year, we have measured monovalent cation transport under different conditions in normal, revertant, and ras-transformed cells. We have focussed on one pathway in particular, the amiloride sensitive Na/H exchanger (antiporter). We find that there is an approximately 50% reduction in the ability of ras-transformed cells to take up ^{22}Na ions by way of the exchanger as compared to nontransformed controls under defined experimental conditions. This significant alteration in Na uptake is accompanied by amiloride-sensitive changes in intracellular pH, again indicating that transformed cells display a marked reduction in the activity of the Na/H exchanger. Revertant cell lines, which are derived from ras-transformed cells but which no longer display all of the characteristics of transformed cells, have "corrected" the deficiency present in transformed cells, but it is not yet clear whether they have restored the activity present in the original NIH/3T3 cells or have other compensatory alterations in monovalent cation transport.

Initial difficulties in isolating DNA sequences responsible for the revertant phenotype in transformed cells are presently being overcome by using retroviral vectors to produce stable multiple transformed cells, by using whole chromosomes to mediate transfer of the revertant phenotype to transformed recipient cells and by using the amino acid 4-cis-L-hydroxy proline as a selective agent that preferentially kills transformed cells.

Publications

Tagliaferri P, Yanagihara K, Ciardiello F, Talbot N, Flatow S, Benade L, and Bassin RH: Effects of Ouabain on NIH/3T3 Cells Transformed with Retroviral Oncogenes, Int. J. of Cancer 40: 653-658 (1987).

Eagen SE, Wright JA, Jarolim L, Yanagihara K, Bassin RH, and Greenberg AH: Transformation by Oncogenes Encoding Protein Kinases Induces the Metastatic Phenotype. Science 238:202-205 (1987).

Talbot N, Hankey P, Jiang B, Yanagihara K, Ciardiello F, Bassin RH, and Benade L: A System for Deriving Revertants of Oncogene Transformed Human Cells. Cyto. and Cell Gen. (in press).

Ciardiello F, Sanfilippo B, Yanagihara K, Kim N, Tortora G, Bassin RH, Kidwell WR, and Salomon DS: Differential Growth Sensitivity to 4-cis-Hydroxy-L-proline Proof Transformed Rodent Cell Lines. Cancer Res. 48: 2483-2491 (1988).

Cooper H L, Bhattacharya B, Bassin RH, and Salomon D S: Suppression of Synthesis and Utilization of Tropomyosin in Mouse and Rat Fibroblasts by Transforming Growth Factor alpha: A Pathway in Oncogene Action. Cancer Res., 47: 4493-4500 (1987).

Wang SY, Bassin RH, and Racker E: Effect of High K^+, Hypertonicity, and Ouabain on MeAIB Uptake and on Growth of C-myc and V-ras Transfected Rat Fibroblasts. Oncogene (1988, in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09003-06 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Alpha Transforming Growth Factors in Rodent and Human Mammary Carcinomas

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

David Salomon	Supv. Res. Biochemist	LTIB, DCBD, NCI
Fortunato Ciardiello	Visiting Fellow	LTIB, DCBD, NCI
Vidya Shankar	Research Fellow	LTIB, DCBD, NCI
Robert Bassin	Chief, Biochemistry Oncogenes Sec.	LTIB, DCBD, NCI
Robert Callahan	Chief, Oncogenetics Sec.	LTIB, DCBD, NCI
Gilbert Smith	Oncogenetics Sec.	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)* Dr. Marc Lippman, Medicine Branch, DCT, NCI
Dr. Nancy Hynes, Ludwig Inst. Cancer Research, Bern, Switzerland
Dr. Ryk Derynck, Dept. of Molecular Biology, Genentech, Inc., San Francisco, CA
Dr. Mary Lou McGeady, Otsuka Pharmaceutical Co., Rockville, MD

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Biochemistry of Oncogenes Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	3	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.)*

Experiments are being conducted to determine the distribution and role of transforming growth factor alpha (TGFα) in normal and malignant rodent and human mammary epithelial cells. Primary dimethylbenz(α)anthracene (DMBA) and nitrosomethylurea (NMU) induced rat mammary adenocarcinomas possess immunoreactive and biologically TGFα . A specific 4.8 Kb TGFα mRNA species could be detected in the DMBA - and NMU - induced rat mammary tumors. Ovariectomy produced a five to tenfold decrease in the expression of TGFα mRNA in the DMBA tumors. Nog 8 mouse mammary epithelial cells transfected with a plasmid containing a glucocorticoid inducible MMTV-LTR linked to the point-mutated c-HA-ras proto-oncogene become transformed following the addition of dexamethesone (dex) to these cells. Following dex treatment, there is a rapid induction of the p21ras protein and of c-Ha-ras mRNA within 3 to 5 hours. During this interval there is a coordinate increase in the level of expression of TGFα mRNA and a subsequent increase in the production of TGFα protein. Human breast cancer cell lines are also producing TGFα and possess TGFα mRNA. In MCF-7 cells, the level of production of TGF α and TGF α mRNA expression can be enhanced by estrogens. Treatment of MCF-7 cells with a polyclonal anti-human TGFα antibody or with a monoclonal antibody against the human EGF receptor will inhibit the growth of these cells in vitro. Elevated levels of TGFα and TGFα mRNA can be detected in approximately 65% of primary human breast tumors. These results suggest that TGFα may function as an autocrine growth factor for a subset of rodent and human mammary tumor cells and that estrogens or activation or over-expression of a ras protooncogene may control the production of this growth factor.

GPO 914-918

Major Findings

We have found that primary dimethylbenz (α) anthracene (DMBA) or nitrosomethylurea (NMV)-induced rat mammary adenocarcinomas possess biologically and immunologically reactive TGFα . A specific TGFα 4.8 kb mRNA species could be detected in these tumors. Following ovariectomy, there was a five - to ten-fold decrease in the level of expression of TGFα mRNA within the DMBA-induced tumors which could be detected within 3 to 6 hours post-ovariectomy. These results demonstrate that the levels of TGFα may be directly correlated with the estrogen-dependent status of these rat mammary tumors. Moreover, the levels of TGFα production may be related to the degree or level of ras gene expression in these tumors since they possess either a point-mutated c-Ha-ras proto-oncogene or elevated levels of c-Ha-ras protein (p21ras) or mRNA. To address this question, we have examined the level of TGFα production and TGFα mRNA expression in a mouse mammary epithelial cell line, Nog 8 cells, which have been transfected with a plasmid containing a human point-mutated c-Ha-ras proto-oncogene ligated to the dexamethasone (dex) -inducible MMTV promoter. Epidermal growth factor (EGF) or TGFα can stimulate the growth of Nog 8 cells in serum-free medium or the anchorage-independent growth of these cells in soft agar. In contrast, dexamethasone-treated Nog 8 cells containing the MMTV-ras expression vector plasmid fail to respond to either exogenous EGF or TGFα . The refractoriness of these cells to these two growth factors is due to the enhanced production of endogenous TGFα following induction of the activated ras proto-oncogene. Within 3 to 5 hours after dex treatment of these cells, there is a four-to five-fold increase in the level of p21ras protein and c-Ha-ras mRNA. Concomitantly, there is a rapid increase in the level of TGF α mRNA expression which is subsequently followed by an enhanced production of TGFα protein which is secreted. These results indicate that TGFα is at least one growth factor that is induced in the presence of an activated ras proto-oncogene in mammary epithelial cells.

TGF- α 's are not restricted to rodent mammary tumors since we have previously demonstrated biologically active αTGFs in the cell membrane from several human breast cancer cell lines and in the acid-ethanol extracts prepared from primary human breast carcinomas. Immunoreactive αTGFs were also detectable in the CM from the breast cancer cell lines, MCF-7, T47-D, ZR-75-1 and MDA-MB-231. In the estrogen responsive cell lines, MCF-7 and ZR-75-1, 17-β estradiol was found to increase the production of αTGFs by two- to three-fold. Biosynthetic labeling of MCF-7 cells with [^{35}S] cysteine followed by immunoprecipitation of the CM and cell extracts with a polyclonal goat anti-human αTGF antibody demonstrated the presence of several high molecular weight αTGF species at 30,000 and 17,000 to 20,000 daltons. Northern blot hybridization of poly A(+)RNA also demonstrated the presence of a 4.8 Kb αTGF mRNA species in these cells using a human αTGF cDNA probe. In detergent extracts prepared from normal breast tissue, a benign fibrocystic lesion, two fibroadenomas and twenty-two primary breast carcinomas, α TGF concentrations were found to range from 1.5 to 6 ng/mg cell protein with approximately 50% of the breast tumors (11/22) possessing α TGF levels greater than 2.5 ng/mg cell protein. Thirty six infiltrating ductal carcinomas were also examined for expression of TGFα mRNA. Seventy percent of these tumors were found to contain a demonstrable 4.8 TGFα mRNA species. The data suggest that TGF α is a growth factor which is frequently expressed in human breast cancer and that TGFα might be useful as an adjunct marker for those tumors which overexpress the ras proto-oncogene.

Recent publications

Salomon DS, Perroteau I, Kidwell WR, Sam J, and Derynck R: Loss of growth responsiveness to epidermal growth factor and enhanced production of alpha transforming growth factors in ras transformed mouse mammary epithelial cells. J Cell Physiol 130: 397-409, 1987.

Liu S, Sanfilippo B, Perroteau I, Derynck R, Salomon DS, and Kidwell WR: Expression of transforming growth fator alpha (TGFα) in differentiated rat mammary tumors: estrogen induction of TGFα production. Mol. Endocrinology 1 (10): 683-692, 1987.

Cooper HL, Bhattacharya B, Bassin RH, and Salomon, DS: Suppression of synthesis and utilization of tropomyosin in mouse and rat fibroblasts by alpha transforming growth factor: a pathway in oncogene action. Cancer Res. 47: 44934500, 1987.

Kidwell WR, Salomon DS, and Mohanam S: Production of growth factors by normal human mammary cells in culture. In: Effect of Human Milk on the Reci pient Infant (eds. A. Goldman and S. Atkinson, L.A. Hanson) Plenum Press, NY. 227-239, 1987.

Kidwell WR, and Salomon DS: Growth factors in human milk: sources and potential physiological roles. In: CRC, Minor Milk Proteins (eds. A. Atkinson and J. Lonnecdol. CRC Press Inc., Boca Raton, FL.) (In press).

Kidwell WR, Mohanam S, Sanfilippo B, and Salomon DS: Tissue organization and cancer: Role of autocrine growth factors in extracellular matrix biosynthesis. In: Proceedings of the 16th Annual UCLA Symposium on Cellular and Molecular Biology. Growth Regulation of Cancer (ed. M.E. Lippman) Alan R. Liss, Inc., NY 1987.

Bates SE, Davidson NE, Valverius E, Dickson RB, Freter CE, Tam JP, Kudlow JE, Lippman ME, and Salomon DS: Expression of transforming growth factor alpha and its mRNA in human breast cancer: Regulation by estrogen and evidence for an autocrine role. Mol. Endocrinology (In press).

Salomon DS, and Kidwell WR: Tumor-associated growth factors in malignant rodent and human mammary epithelial cells. In Breast Cancer: Cellular and Molecular Biology. Vol. II (eds. M.E. Lippman and R. B. Dickson) Academic Press, New York. (In press).

Bhattacharya B, Ciardiello F, Salomon DS, and Cooper HL: Disordered metabolism of microfilament proteins, tropomyosin and actin, in mouse mammary epithelial cells expressing the Ha-ras oncogene. Oncogene Research (In press).

Ciardiello F, Sanfilippo B, Yanagihara K, Kim N, Tortora G, Bassin RH, Kidwell WR, and Salomon DS: Differential growth sensitivity to 4-CIS-hydroxy-L-proline of transformed rodent cell lines. Cancer Res. (In press).

Sanfilippo B, Ciardiello F, Salomon DS, and Kidwell, WR: Growth of cells on a perfluorocarbon medium interphase: a quantitiative assay for anchorage independent cell growth. In Vitro. 24: 71-78, 1988.

Kidwell WR, Mohanam S, and Salomon DS: Growth factor production by mammary tumor cells. In: Molecular Biology of Breast Cancer (eds. D. Medina, WR Kidwell, C. Ip, and E. Andersen), Academic Press, NY (In press).

Salomon, DS, Kidwell WR, Kim N, Ciardiello F, Bates SE, Valverius, E, Lippman ME, Dickson RB, and Stampfer M: Modulation by estrogen and growth factors of transforming growth factor alpha (TGFα) expression in normal and malignant human mammary epithelial cells. Recent Advances Cancer Res. (In press).

Mohanam S, Salomon DS and Kidwell WR: Substratum modulation of epidermal growth factor receptor expression by normal mouse mammary cells. J. American Dairy Science Assoc. (In press).

Ciardiello F, Kim N, Hynes N, Jaggi R, Redmond S, Liscia DS, Sanfilippo B, Merlo G, Callahan R, Kidwell WR and Salomon DS: Induction of transforming growth factor alpha expression in mouse mammary epithelial cells after transformation with a point-mutated c-Ha-ras protooncogene. EMBO Journal (In press).

Valverius E, Bates SE, Stampfer MR, Clark R, McCormick F, Salomon DS, Lippman ME and Dickson RB. Transforming growth factor alpha and its receptor in human mammary epithelial cells: modulation of epidermal growth factor receptor function with oncogenic transformation. Mol. Endocrinology. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05148-09 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
Gilbert Smith	Microbiologist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. John Silver, LVD, NIAID, NIH

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Oncogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

We have previously described a common integration site (designated int-3) for MMTV in virus-induced mouse mammary tumors. MMTV integration at this locus activates the expression of a 6.6- and 2.4-kb species of RNA. These RNA species correspond to flanking cellular genomic sequences that are located 5' and 3', respectively, of the integrated viral genome. We are currently analyzing cDNA recombinant clones of the 2.4-kb species of int-3 RNA. In other studies, we have shown that there is a significant difference in the frequency with which MMTV (C3H) integrates at int-1 in BALB/cfC3H compared with C3H mammary tumors. Similarly, there is a significant difference in the frequency of MMTV (RIII) integration events at int-2 in BALB/cfRIII compared with RIII mammary tumors. These results suggest that the inbreeding program for high tumor incidence in C3H and RIII mouse strains has selected for host genetic mutations that affect the frequency of int gene activation. To begin to dissect at a genetic level the mutational events resulting in the development of preneoplastic and malignant mammary epithelial phenotypes, we have developed several mammary hyperplastic outgrowth lines (HOGs) in CZECHII V^+ mice. These HOGs and primary mammary epithelia are currently being used as targets for retroviral vectors containing the int genes and other oncogenes.

GPO 914-918

Major Findings

I. The int-3 locus in MMTV-induced mammary tumors in CZECHII mice

We have previously shown that 8 of 44 mammary tumors in CZECHII mice contain a viral insertion at the int-3 locus. In each case, the transcriptional orientation of the viral genome was in the same direction. Viral insertion at the int-3 locus activates expression of two cellular RNA species, 6.6 and 2.4 kb, respectively. The 6.6-kb RNA species corresponds to host cellular sequences flanking the 5' end of the integrated viral genome. The 2.4-kb RNA species corresponds to flanking host cellular sequences 3' to the integrated viral genome. Nucleotide sequence analysis of the 5' host flanking cellular sequences has revealed a translational long open reading frame in the opposite transcription orientation relative to the integrated viral genome. We are currently analyzing the nucleotide sequence of recombinant cDNA clones of the 2.4-kb RNA species and obtaining recombinant clones of the 6.6-kb RNA species.

II. The frequency of MMTV insertion at the int loci is a function of the host genetic background

Published reports by several laboratories have shown that the frequency with which MMTV integrates at int-1, int-2, and int-3 in mammary tumor DNA differs depending on the mouse strain. Using foster nursing, we have introduced the MMTV(C3H) and MMTV(RIII) virus strains onto the low tumor incidence BALB/c mouse strain. We have found: 1) a significant decrease ($P \leq 0.002$) in the frequency of MMTV integrations at int-1 in BALB/cfC3H compared with C3H mammary tumors; and 2) a similar decrease ($P \leq 0.026$) in the frequency of MMTV integrations at int-2 in BALB/cfRIII compared with RIII mammary tumors. These results suggest that inbreeding for high tumor incidence in C3H and RIII mouse strains has resulted in the fixation of host cellular mutations. These mutations could affect the frequency of viral integration at int-1 and int-2 by either: 1) complementing the biological effect of the respective int gene activation in mammary tumorigenesis or 2) affecting the sensitivity of clonal subpopulations of developing mammary epithelial cells that are sensitive to expression of int-1 or int-2 gene activation. Since only 50-60% of the BALB/cf mammary tumors contained a viral integration within int-1, -2, or -3, there are probably additional as yet unidentified int loci.

III. Molecular biology of premalignant lesions

Transplantation studies demonstrate that epithelial stem cells must exist in the mouse mammary gland throughout life. These cells are present throughout the gland and possess characteristics, evident in explant culture, of undetermined mammary epithelial cells. We are investigating the role of these cells and their progeny in the development of serially transplantable hyperplastic epithelial outgrowth lines in MMTV provirus-free CZECHII mice and in primary in vitro cultures of mammary cells capable of repopulating epithelium-free mammary fat pads. It is our intention to introduce, via retroviral expression vectors, various mammary-specific oncogenes and putative oncogenes such as int-1, -2, and -3 into primary cultures of both normal and preneoplastic mammary epithelial cell populations in vitro precedent to repopulation of the epithelium-free mammary fat pad. We hope to delineate the direct and/or cooperative effects of these genes

in the establishment of the premalignant (nonsenescent) or malignant mammary epithelial phenotype.

Publications

Callahan R. Retrovirus and proto-oncogene involvement in the etiology of breast neoplasia. In: Neville MC, Daniel C, eds. The mammary gland: development, regulation and function. New York: Plenum Publishing Corp., 1987;323-51.

Dean M, Kozak C, Robbins J, Callahan R, O'Brien SO, Van de Woude GF. Chromosomal localization of the met proto-oncogene in the mouse and cat genome. Genomics 1987;1:167-73.

Reeves RH, Gallahan D, O'Hara BF, Callahan R, Gearhart JD. Genetic mapping of Prm-1, Igl-1, Smst, Mtv-6, Sod-1 Ets-2, and localization of the Down syndrome-region on mouse chromosome 16. Cytogenet Cell Genet 1987;44:76-81.

Smith GH. Functional differentiation of virgin mouse mammary epithelium in explant culture is dependent upon extracellular proline. J Cell Physiol 1987; 131:190-9.

Smith GH. Overt and cryptic functions of the mouse mammary tumor virus long terminal repeat DNA sequence. In: Medina D, Kidwell W, Heppner G, Anderson E, eds. Cellular and molecular biology of mammary cancer. New York: Plenum Press, 1987;335-51.

Smith GH. In situ detection of transcription in transfected cells using biotin-labeled molecular probes. Molecular genetics of mammalian cell. In: Gottesman, M. (Ed.) Academic Press, Miami. Methods Enzymol (Colowick S.P. and Kaplan) 1987; 151:530-8.

Smith GH, Medina D. A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. J Cell Sci 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04829-14 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 charecters 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 Associate	LTIB, DCBD, NCI
Georgio Merlo	Visiting Fellow	LTIB, DCBD, NCI
Danielle Liscia	Visiting Associate	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

D. Carleton Garrett, George Washington University Medical School, Washington, DC
Dr. Rosette Lidereau, Rene Huguenin Centre, St. Cloud, France

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Oncogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.4	3.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither A/B

☐ (a1) Minors

☐ (a2) Interviews

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

We have continued our efforts to identify and characterize frequent genetic changes associated with primary human breast tumor DNAs. The int-2 gene is related to the fibroblast growth factor gene family. It is frequently activated by mouse mammary tumor virus-induced insertional mutagenesis in mouse mammary tumors. We have found that int-2 is amplified 2- to 15-fold in 16% of human breast tumors (n=118). This genetic alteration has a highly significant association ($P \leq 2 \times 10^{-6}$) with patients who subsequently developed a local recurrence or distal metastasis. The c-erbB-2 gene is related to the gene encoding the epidermal growth factor (EGF) receptor. We found that 10% of the breast tumor DNA contain an amplification of c-erbB-2. In contrast to one published report from another laboratory, we did not find a significant association between this mutation and the patients' prognosis.

PHS 6040 (Rev. 1/84)

GPO 914-916

Major Findings

In previous work on frequently occurring mutations in primary human breast tumor DNA, we found the c-myc proto-oncogene amplified in 40% of the tumors. We have extended this study to other proto-oncogenes and have found that the int-2 and c-erbB-2 proto-oncogenes are also amplified in different subsets of the tumor DNA panel. The int-2 gene is related to the gene encoding basic fibroblast growth factor (bFGF). Its expression is activated by MMTV insertional mutagenesis in mouse mammary tumors. We have found that in 16% of the primary human breast tumor DNAs (n=118), the int-2 gene is amplified 2- to 15-fold. Amplification of int-2 has a highly significant association ($P \leq 2 \times 10^{-6}$) with patients who later develop a local recurrence or distal metastasis. Since amplification of int-2 is not associated with the lymph node status of the patient, this may represent an additional useful clinical test to determine the patient's prognosis. We are currently determining whether amplification of int-2 is associated with activation of int-2 RNA expression, as well as whether other closely linked proto-oncogenes are co-amplified with int-2.

The c-erbB-2 gene is related to the epidermal growth factor receptor (EGFR) gene. Others have reported that amplification of this gene in primary breast tumor DNA has a highly significant correlation with the patient's prognosis. In our panel of breast tumor DNAs (n=110), 10% contained an amplification of c-erbB-2. However, there was no significant association between c-erbB-2 amplification and the patients' prognosis.

Publications

Ali IU, Lidereau R, Theillet C, Callahan R. Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. Science 1987;238:185-8.

Lidereau R, Callahan R, Dickson C, Peters G, Escot C, Ali I. Amplification of the int-2 gene in primary human breast tumors. Oncogene Res 1988;2:285-91.

Mariani-Costantini R, Escot C, Theillet C, Gentile A, Merlo G, Lidereau R, Callahan R. In situ c-myc expression and genomic status of the c-myc locus in infiltrating ductal carcinomas of the breast. Cancer Res. 1988; 48:199-205.

Callahan R. Retrovirus and proto-oncogene involvement in the etiology of breast cancer. In: Neville MC, Daniel C, eds. The mammary gland: development, regulation and function. New York: Plenum Press, 1988;in press.

Callahan R, Weeks MO, Ohuchi N, Schlom J. The potential involvement of retroviral related genes and proto oncogenes in breast neoplasia. In: Litwin SD, Scott D, Flaherty L, Reisfeld R, Marcus P, eds. Human immunogenetics: an advanced text. New York: Marcel Dekker, Inc., 1988;in press.

Ali IU, Lidereau R, Callahan R. Heterogenicity of genetic alterations in primary human breast tumors. In: Lippman ME, Dickson R, eds. Breast cancer: cellular and molecular biology. Boston: Martinus Nijhoff Publishing, 1988;in press.

Callahan R. Two families of human endogenous retroviral genomes in eucaryotic transposable elements as mutagenic agents. In: Banbury reports. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1988;in press.

Lynch HT, Watson P, Marcus JN, Merlo G, Callahan R, Schlom J, Conway T, Fitzsimmons ML, Lynch J: Hereditary breast cancer: search for biomarkers. J Tumor Marker Oncol 1988;in press.

Ali IU, Campbell G, Lidereau R, Callahan R. Amplification of c-erbB-2 proto-oncogene in primary human breast tumors: lack of evidence for a strong association with aggressiveness of the disease. Science 1988;in press.

Ali IU, Campbell G, Lidereau R, Callahan R. Lack of evidence for the prognostic significance of c-erbB-2 amplification in human breast carcinoma. Oncogene Res. 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 09023-02 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Cloning of Immunoglobulin Genes to Tumor Antigens

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

Syed Kashmiri	Expert	LTIB, DCBD, NCI
Robert Callahan	Chief, Oncogenetics Section	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI
Jim Xiang	Visiting Fellow	LTIB, DCBD, NCI
Anna Maria Masci	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. Mark Bodmer, Celltech, Inc., England

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Oncogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.3	3.3	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Our research effort is aimed at designing and constructing recombinant immunoglobulin genes derived from hybridomas releasing monoclonal antibodies (MAbs) against human tumor-associated antigens. The purpose of this effort is to modify the MAbs to: 1) reduce their immunogenicity in patients, 2) enhance the pharmacokinetics of the MAbs in patients, and 3) increase the binding of the MAb for the target antigen. We have obtained recombinant clones of genomic DNA containing the rearranged variable regions of the heavy and light chain genes from the 72.3 hybridoma. The nucleotide sequence of the light chain gene has been determined. In addition, the IgK VJ sequences have been inserted into an expression vector containing human Ig constant region. Cells transfected with this vector express human-mouse chimeric 72.3 Ig. Similar experiments are underway with the 72.3 Ig VDJ-containing recombinant clone.

PHS 6040 (Rev. 1/84)

GPO 914-916

Major Findings

Recombinant clones of rearranged genomic immunoglobulin genes encoding the heavy and light chain variable regions of the 72.3 MAb have been obtained. The nucleotide sequence of the 72.3 VDJ κ chain VJ regions have been determined. Restriction fragments corresponding to both variable regions have been inserted into expression vectors containing the respective human C-γ1 or κ chain constant regions. These recombinant constructs have been transfected singly and in combination into a nonimmunoglobulin-producing myeloma cell line. Chimeric κ chain production has been detected by Western blot analysis of transfectoma cell extracts. Analysis of transfectomas expressing chimeric 72.3 heavy chain and H_2L_2 production are in progress. Preparation of recombinant clones of the chimeric IgG- γ1 and genes in which CH domains have been deleted has been initiated. In other work, we have obtained recombinant cDNA clones of other MAbs which react with the TAG-72 antigen.

Publications:

Whittle N, Adair J, Lloyd C, Jenkins L, Devine J, Schlom J, Raubitschek A, Colcher D, and Bodmer M. Expression in COS cells of a mouse-human chimaeric B72.3 antibody, Protein Engineering 1987;1:499-505.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05216-17 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Cyclic AMP (cAMP) in Growth Control of Neoplasia

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
D. Katsaros	Visiting Fellow	LTIB, DCBD, NCI
G. Tortora	Visiting Fellow	LTIB, DCBD, NCI
S. Ally	Guest Worker	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. W. R. Miller, U. of Edinburgh
Dr. L. Neckers, LP, DCBD, NCI
Dr. S. O. Doskeland, U. of Bergen, Norway
Dr. R.K. Robins, Nuclei Acid Research Institute, Costa Mesa, CA

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	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.)*

Cyclic AMP (cAMP) in mammalian cells functions by binding to cAMP receptor protein, the regulatory subunit of cAMP-dependent protein kinase. The cAMP receptor protein has two different cAMP binding sites, and cAMP analogs that selectively bind to either one of the two binding sites are known as Site 1-selective (C-2 and C-8 analogs) and Site 2-selective (C-6 analogs), respectively. Such site selective cAMP analogs at micromolar concentrations exert potent growth inhibition with no sign of toxic effects in a spectrum of human cancer cell lines, including those that are resistant to cAMP analogs tested previously, leukemia lines, and v-rasH oncogene-transformed NIH/3T3 cells; the combination of a C-8 with a C-6 analog showed synergistic effects. The growth inhibition was not due to a block in a specific phase of cell cycle but paralleled selective modulation of type I versus type II protein kinase isozymes, suppression of cellular proto-oncogene expression, phenotypic change, and differentiation (leukemic cells). It is suggested that the site-selective cAMP analogs, substituting for endogenous cAMP and binding to protein kinase, suppress the cancer cell growth not via directly inhibiting cell division but rather by promoting cell differentiation. Site-selective cAMP analogs thus provide new biological tools for investigating cell proliferation and differentiation and also for the improved management of human cancer.

GPO 914-918

Major Findings

New site-selective cAMP analogs demonstrated the growth inhibition with many-fold greater potency than previously studied analogs, in a spectrum of 20 human cancer cell lines. Of 24 site-selective analogs tested, the majority produced growth inhibition (30-80%) with no sign of toxicity in all 20 cancer cell lines at micromolar concentrations. The growth inhibition was not due to a block in cell cycle progression but paralleled a change in cell morphology, an augmentation of the RII cAMP receptor protein, and a decrease of p21 ras protein. Since the adenosine counterpart of the analog produced cell death and G1 synchronization without affecting the RII and p21 ras protein levels, it is unlikely that an adenosine metabolite is involved in the analog effect. The effect of site selective cAMP analogs on the growth of rat mammary tumors and cancer cells implanted in athymic mice was also tested. These in vivo models were used to determine the efficacy as well as toxicity of cAMP analogs. Studies demonstrated that the site-selective analogs that are most potent in the growth inhibition of the cancer cell lines in culture are also most effective in producing tumor regression in vivo. Site-selective cAMP analogs thus provide a biological tool for control of malignant growth.

Publications

Ogreid D, Cho-Chung YS, Ekanger R, Vintermyr O, Haavik J, and Doskeland SO: Characterization of the cAMP effector system in hormone-dependent and hormone-independent rat mammary carcinomas. Cancer Res 47 2576-2582, 1987.

Cho-Chung YS: Models of tumor regression in endocrine related cancer In: Endocrine Management of Cancer. Vol 1. B.A., Stoll (Ed.), S., Karger, A., Basel, Switzerland. 1-13, 1988.

Cho-Chung YS: Site-selective cAMP analogs in the arrest of cancer cell growth. In: Developments in Cancer Chemotherapy Vol II. R. Glazer (Ed.), CRC press, Boca Raton, FL (In press).

Katsaros D, Tortora G, Tagliaferri P, Clair T, Ally S, Neckers L, Robins RK, and Cho-Chung YS: Site-selective cyclic AMP analogs provide a new approach in the control of cancer cell growth. FEBS Lett 223, 97-103, 1987.

Tagliaferri P, Katsaros D, Clair T, Ally S, Tortora G, Neckers L, Rubalcava B, Parandoosh Z, Chang YA, Revankar GR, Crabtree GW, Robins RK, and Cho-Chung YS: Synergistic inhibition of growth of breast and colon human cancer cell lines by site-selective cAMP analogs. Cancer Res. 48, 1642-1650, 1988.

Tagliaferri P, Katsaros D, Clair T, Necker L, Robins RK, and Cho-Chung YS: Reverse transformation of Ha-MuSV-transformed NIH/3T3 cells by site selective cAMP analogs. J. Biol. Chem 263, 409-416, 1988.

Clair T, Ally S, Tagliaferri P, Robins, RK, and Cho-Chung YS: Site-selective cAMP analogs induce nuclear translocation of the RII cAMP receptor protein in Ha-MuSV-transformed NIH/3T3 cells. FEBS Lett. 224, 377-384, 1987.

Tortora G, Tagliaferri P, Clair T, Colamonici O, Neckers LM, Robins RK, and Cho-Chung YS: Site-selective cAMP analogs at micromolar concentrations induce growth arrest and differentiation of acute promyelocytic, chronic myelocytic, and acute lymphocytic human leukemia cell lines. Blood, 71 230-233, 1988.

Katsaros D, Ally S, and Cho-Chung YS: Site-selective cyclic AMP analogs are antagonistic to estrogen stimulation of growth and proto-oncogene expression in human breast cancer cells. Int. J. Cancer (In press).

Cho-Chung YS: Models of tumor regression in endocrine-related cancer. In: Reviews on Endocrine - Related Cancer (In press).

Ally S, Tortora G, Clair T, Grieco D, Merlo G, Katsaros D, Ogreid D, Doskeland SO, Jahnsen T, and Cho-Chung YS: Selective modulation of protein kinase isozymes by site-selective 8-cl-cAMP provides a biological means for control of human colon cancer cell growth. Proc. Natl. Acad Sci (In press)

Cho-Chung US, Clair T, Tagliaferri P, Ally S, Katsaros D, Tortora G, Neckers L, Avery T, Crabtree G, and Robins RK: Site-selective cAMP analogs as a new biological tool in growth control, differentiation, and proto-oncogene regulation-basic science review. Cancer Invest (In press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08281-06 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

cAMP Receptor Protein in Gene Transcription

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
G. Tortora	Visiting Fellow	LTIB, DCBD, NCI
T. Clair	Chemist	LTIB, DCBD, NCI
S. Ally	Guest Worker	LTIB, DCBD, NCI
R. Bassin	Chief, Biochemistry Oncogenes Section	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. J. D. Corbin, Howard Hughes Medical Inst. Lab. Vanderbilt, U., Nashville, TN
Tore Jahnsen, Institute of Pathology, Rikshospitalet, Oslo, Norway
Dr. H. Fan, University of California, Irvine, CA

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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.)*

In prokaryotes, cAMP is known to regulate the transcription of specific genes by a well-defined mechanism. cAMP binds to the prokaryotic cAMP receptor protein (CRP), and this complex interacts with specific DNA sequences at the 5' ends of targeted genes, modulating the ability of RNA polymerase to bind to the promoters. cAMP has also been shown to alter the transcription of specific eukaryotic genes. Recent evidence suggests a direct action of cAMP on mammalian genes in which the regulatory subunit (R) of cAMP-dependent protein kinase might interact directly with target genes. Nagamine and Reich proposed that the R subunit of cAMP-dependent protein kinase could be envisaged as a cAMP- and DNA-binding molecule which regulates gene transcription, the vertebrate counterpart of bacterial CRP. However, this hypothesis lacked evidence for a direct interaction between DNA and cAMP-dependent protein kinase or the R subunit. In this study, we examined the role of cAMP receptor protein, R, in gene transcription. Since cAMP-dependent protein kinase is exclusively present in the cytoplasm, any nuclear function of the protein kinase must be accompanied by its translocation to the nucleus. It was found that the ras gene suppression, growth inhibition, and phenotypic reversion of Ha-MuSV-transformed NIH/3T3 cells by site-selective cAMP analogs correlated with the nuclear translocation of the RII cAMP receptor protein, the regulatory subunit of protein kinase type II. Within 30 min after treatment of the transformed cells with the analogs, immunofluorescence against the RII protein markedly increased in the cell nucleus. Thus, the nuclear translocation of the RII cAMP receptor protein is an early event in the reverse transformation induced by the cAMP analogs. The goal of this study is to provide direct evidence of interaction between the cAMP consensus sequences and cAMP receptor protein (RII) and/or a phosphoprotein substrate of protein kinase.

GPO 914-918

Major Findings:

Site-selective cAMP analogs, depending on the position of their substituents on the adenine ring, selectively bind to either Site 1 or Site 2 of the known cAMP binding sites of protein kinase. Treatment of Harvey murine sarcoma virus-transformed NIH/3T3 cells with such site-selective analogs resulted in growth inhibition and phenotypic reversion, and the combination of a C-8 thio or halogen analog (Site 1-selective) with a N6 analog (Site 2-selective) produced a synergistic effect. The growth inhibitory effect of the analogs correlated with the nuclear translocation of the RII cAMP receptor protein, the regulatory subunit of protein kinase type II. The transformed NIH/3T3 cells contained no detectable level of RII in the nucleus, whereas nontransformed NIH/3T3 cells exhibited a high level of nuclear RII. Within 30 min after treatment of the transformed cells with the site-selective analogs, immunofluorescence against the RII protein markedly increased in the cell nucleus. These results indicate that the nuclear translocation of the RII cAMP receptor protein is an early event in the reverse transformation of the fibroblasts treated with site-selective cAMP analogs.

Publications:

Clair T, Ally S, Tagliaferri P, Robins RK, and Cho-Chung YS:
Site-selective cAMP analogs induce nuclear translocation of the RII cAMP receptor protein in Ha-MuSV-transformed NIH/3T3 cells. FEBS Lett. 224, 377-384, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08280-05 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Cellular Proto-Oncogene Expression and Malignancy

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
S. Ally	Guest Researcher	LTIB, DCBD, NCI
D. Katsaros	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

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

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	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.)*

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-rasH in 70% of estrogen and progesterone receptor positive tumors and 40% of estrogen and progesterone receptor negative tumors. Whereas, an amplified or rearranged crasH gene has not been detected in human mammary carcinomas. Thus, the mechanism by which c-rasH gene expression is deregulated in these tumors remain to be determined. The goal of this project is to elucidate the mechanisms of oncogene involvement in the neoplastic transformation and progression.

Major Findings

The p21 RAS PROTEIN EXPRESSION BY HUMAN BREAST CANCER SHOWS PROGNOSTIC SIGNIFICANCE. An enhanced expression of p21 protein has been demonstrated in the majority of human mammary carcinomas utilizing immunohistochemical technique and Western Blotting analysis and elevation of c-rasH mRNA in human breast tumors has been demonstrated using dot-blot hybridization technique. No studies to date, however, have been reported on the relationship between the p21 levels and disease state of breast cancer. The objective of this study was to examine whether the increased production of p21 is associated with progression and prognosis of breast cancer. Expression of p21 ras protein was measured in tumors from patients who had nonmetastasized breast cancer. Western Blotting analysis revealed that 42 of 57 (74%) tumors contained p21 levels 2- to 10-fold greater than those of control tissues. An excessive increase of p21 (5- to 10-fold over the control value) occurred more frequently in tumors of T3 and T4 stages (15 of 27 [56%]) than in tumors at T2 stage (9 of 30 [30%]) suggesting a correlation between advancement of disease and high p21 levels. p21 levels were positively related to the presence of estrogen receptors and also involvement of axillary lymph nodes with tumor. As no correlations were detected between p21 levels and tumor cellularity or grade, it is possible that p21 levels may reflect the degree of cellular malignancy. This is supported by data on tumor recurrence, 13 of 16 patients (81%) with tumors expressing low p21 levels were disease free for $\geq$ 4 years after primary treatment, whereas only 5 of 10 patients (50%) with high p21 tumors remained disease free. These results suggest that a quantitative enhancement of p21 oncogene protein is associated with both the progression and poor prognosis of breast cancer.

Publications

Clair T, Miller WR, and Cho-Chung YS: Prognostic significance of the expression of a ras protein with a molecular weight of 21,000 by human breast cancer. Cancer Res. 47, 5290-5293, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08249-07 LTIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
Sue Liu	Bio. Lab.Tech	LTIB, DCBD, NCI
Brunella SanFilippo	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Rick Derynck	Genentech
Richard Grosse	Akadamie der Wissenschaffen der DDR
Daryl Liu	Bureau of Biologics, FDA

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cell Cycle Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Mechanisms for the escape of breast cancer cells from normal growth controls are being evaluated in human and rodent tissues. Two growth inhibitors and two growth stimulatory factors have been isolated from breast tissues, breast fluids, and/or breast cell lines. Two of the factors were shown to be transforming growth factor alpha (TGF) and transforming growth factor beta (TGF). The former was made by normal breast cells and by some tumors. Its synthesis appeared to be regulated by estrogenic hormones. The latter factor was synthesized in similar amounts by normal and neoplastic mammary epithelium. Two other growth modulators isolated from human breast tissues and/or milk represent activities not described by other groups. One was mammary-derived growth factor I (MDGFI), a 62-kD peptide that greatly enhanced normal mammary cell proliferation and basement membrane synthesis. MDGFI was purified to apparent homogeneity and its N-terminal sequence determined. There was no homology with any known protein. The fourth factor, mammary inhibitory factor (MIA) was also purified to apparent homogeneity and sequenced. MIA was shown to be 95% homologous with the N-terminal sequence of α-lactalbumin. Both normal and cancer cell lines from human breast were inhibited by MIA; in general the normal cells were more sensitive to it. Alpha-lactalbumins from several species (cow, human, monkey, mouse, rat, and camel) were all inhibitory at low concentrations (1-10 ng/ml).

GPO 914-918

Major Findings

1. Mammary Derived Growth Factor (MDGFI). MDGFI was purified to apparent homogeneity by isoelectric focusing, gel permeation, and reverse phase HPLC chromatography. The factor is a Mr 62,000 protein with a pI of 4.8. ^{125}I-labeled MDGFI was found to bind to high affinity membrane sites on mammary and other cell types. The factor stimulated mammary cell growth optimally at 2.5 ng/ml growth medium and differentially stimulated the cells to produce type IV collagen. The possibility that MDGFI was synthesized by human mammary epithelium was assessed by analysis of conditioned medium from primary cultures of normal and malignant human mammary cells for activity that competed with purified 125I-labeled MDGFI for binding to mammary cell receptors. Competing activity was found from both sources, but when normalized to the number of cells present in the primary cultures, the tumor cells were found to secrete about two to three times as much MDGFI as normal cells. MDGFI was sequenced from the N-terminal end. A computer search failed to reveal any homology with known proteins. Thus, MDGFI appears to be a new growth factor. Frozen preparations of partially purified MDGFI, which were originally growth stimulatory, became growth inhibitory. These observations led to the discovery of MIA, or mammary inhibitory activity.

2. Mammary Inhibitory Activity (MIA). MIA has been purified to homogeneity by means of isoelectric focusing, molecular sieve chromatography (FPLC), and electroelution from acrylamide gels. The protein has been shown by immunodot blotting and Western blotting to be immunologically related to but not the same as growth inhibitors purified from bovine milk by Grosse and one isolated from cultures of mouse fibroblasts by Wang. The factor inhibits normal mammary cell growth by about 80% at a concentration of 10 ng/ml. At the same concentration, collagen IV synthesis is reduced by 90%. These effects of MIA were not receptor mediated, since no evidence for receptors on mammary cells was observed. MIA has been sequenced and shown to be 95% homologous to human α -lactalbumin.

3. Transforming Growth Factor Alpha (TGFα). Previous studies revealed that TGF α was produced by primary rodent mammary adenocarcinomas, as well as by normal mammary cells, including those from human tissue. About 50% of the human tumors were found to possess TGFα mRNA. Of these, 75% were positive for both estrogen and progesterone receptors. Similar results were found in the distribution of TGFα mRNA in rodent mammary tumor models. Direct demonstration of the regulation of TGF α expression was made in cell cultures of rodent mammary adenocarcinomas. Cells cultured in the absence of estradiol lost all TGFα mRNA within 3 days. However, if the steroid was present, mRNA levels were readily detected. Since there was a correlation between TGFα production and basement membrane synthesis by rodent tumors, TGFα effects on basement membrane synthesis were assessed. At 10 ng/ml, collagen IV synthesis was elevated about 4-fold. Additionally, since estrogen stimulated the production of TGFα , the effect of estradiol on collagen IV was measured. A 2-fold elevation was seen. It thus appears that basement membrane synthesis may be positively regulated by MDGFI and by TGFα both produced by the mammary epithelium itself.

4. Transforming Growth Factor Beta (TGFβ). TGFβ production by normal and malignant human mammary epithelium was assessed by bioassay of conditioned medium from primary cultures of these cells for soft agar growth-promoting activities in the presence of saturating amounts of TGFβ . Very large amounts of TGFβ activity

were detected (up to 20 ng/ml conditioned medium). When normalized against the number of cells present in the cultures, the normal human mammary cells were found to produce as much TGFβ as the tumor cells. These results indicate that TGF β is, by itself, probably not a significant factor in the control of normal or neoplastic mammary cell growth. However, changes in TGFα (or MDGFI) might alter the potential for TGFβ -mediated growth control, especially since TGFα negates the negative regulatory effects seen with TGFβ alone.

Publications

Liu S, Sanfilippo B, Perroteau I, Derynck R, Salomon DS, Kidwell WR. Expression of TGFα in differentiated rat mammary tumors: estrogen induction of production. Mol Endocrinol 1987;I:683.

Sanfilippo B, Salomon DS, Kidwell WR. Growth of cells on a perfluorocarbon-medium interphase: a quantitative assay for anchorage-independent cell growth. In Vitro 1988;24:71.

Ciardiello F, Sanfilippo B, Yangihara K, Kim N, Tortora G, Bassin R, Kidwell WR, Salomon DS: Differential growth sensitivity to cis-hydroxyproline of transformed rodent cell lines. Cancer Res 1988;48:2483.

Kidwell WR. Assays of growth factor stimulation of extracellular matrix biosynthesis. Methods Enzymol 1987;147:407-14.

Mohanam S, Salomon DS, Kidwell WR. Effects of extracellular matrix on expression of EGF receptors by normal mammary epithelium. Dairy Sci 1988;47:118.

Annual Summary Report, Laboratory of Cell Biology
National Cancer Institute
October 1, 1987 - September 30, 1988

The following are selected highlights of accomplishments for the Laboratory of Cell Biology for the fiscal year 1988.

Three tumor-specific transplantation antigens (TSTA) have been purified from the Meth A tumor. Our laboratory has described two of these antigens one of which has been shown to be a major cytosolic heat shock protein (hsp) consisting of two isoforms of 84 and 86 kDa and the other a less abundant cytosolic but highly immunogenic 82 kDa protein also present in nontransformed cells. Recently, the Sloan-Kettering group have purified a third TSTA of 96 kDa and have isolated a corresponding partial genomic clone, but they were unable to identify it as a previously defined protein. We have now identified the nature of the protein, using a synthetic peptide antiserum against the 96 kDa antigen as the 97 kDa glucose regulated protein (GRP), a member of the 84 and 86 kDa hsp family. This is most likely the 96 kDa TSTA and biologic assays are now in progress with DeLeo (Pittsburgh) to confirm this. All of the Meth A TSTAs thus have counterparts in nontransformed cells which are indistinguishable by molecular mass, isoelectric point or by the individual anti-TSTA sera from the corresponding Meth A TSTA.

The genes coding for both the 84 and 86 kDa hsp-related TSTAs have been isolated from the Meth A tumors. A full length cDNA for the Meth A 84 kDa hsp was previously sequenced; it is an acidic protein of 724 amino acid residues. DNA sequences homologous to hsp 84 were previously mapped to three different chromosomes: 17, 12 and 2. Using specific oligonucleotide probes, the active gene was mapped to chromosome 17. The chromosomal location of hsp 84 relative to the MHC class I region is currently being mapped. The chromosome 17 hsp 84 gene from nontransformed murine cells is now complete in the protein coding region, no differences between it and the Meth A hsp 84 were found in this region. The gene is located on about 6 kb of DNA and is interrupted by 10 introns. Full length cDNA has also been sequenced for the 86 kDa hsp from Meth A tumors; it is an acidic protein of 733 amino acid residues. The 84 and 86 kDa hsp isoforms are 80% homologous. The protein coding regions are co-linear except for an insertion of 9 amino acids near the N-terminus of hsp 86. DNA sequences related to hsp 86 have been assigned to chromosomes 12, 11, and 3. The gene coding for hsp 86 from normal mouse cells is currently being isolated. Comparison of the normal and Meth A sequences is expected to reveal any changes which may have occurred between this TSTA and its normal cellular counterpart.

It has been previously shown by immunofluorescence and immune electron microscopy, using affinity purified antisera, that the 82 kDa and the hsp related TSTAs are present in the cytoplasm and at the cell surface of *in vitro* grown cell cultures, however, these experiments did not indicate whether or not these antigens are present at the cell surface as intact molecules or partially degraded polypeptides or the mechanism whereby intracellular proteins are expressed at the cell surface. In order to define the molecular weight of the antigens present at the cell surface of Meth A cells and to extend these observations to Meth A tumors *in vivo*, the expression of these antigens was examined using the ^{125}I/lactoperoxidase technique to radiolabel cell surface molecules of tumor cells grown *in vitro* and *in vivo*. All three Meth A TSTAs could be immunoprecipitated from cell surface radiolateled *in vivo* or *in vitro* lysates using the individual anti-TSTA sera. The level of the 82 kDa TSTA and the 84 and 86 kDa TSTAs increased approximately 2-fold

in in vivo compared to in vitro Meth A cells. The level of the 96 kDa TSTA was essentially unchanged between in vivo and in vitro cells. In addition to the intact hsp 84 and 86, the anti-hsp sera immunoprecipitated several lower molecular weight species ranging from 75 to 35 kDa; thse apparently represent degradation products of the 84 and 86 kDa TSTAs. The radiolabeled lysates were also tested for the presence of another intracellular protein, hsp 70, and, as was found for the 82 kDa and the 84/86 kDa TSTAs, a 2-fold increase was seen in in vivo cell lysates. Furthermore, ^{35}S-methionine-labeled lysates indicated that other normally intracellular proteins such as actin and GRP 78 wre also present at the cell surface of Meth A cells. These data along with the immunofluorescence and E.M. results suggests that a likely mechanism for the presence of the cytosolic 82 kDa and the 84/86 kDa TSTAs at the cell surface is due to cell lysis followed by binding of these relatively abundant proteins to the cell surface.

Investigations continue relating to: 1) the host immune response to malignant melanoma and the role it plays in the progression of tumor growth and metastatic spread; 2) tumor specific proteins produced by melanoma cells in vivo and in vitro 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 melanoma; 3) the role of cell surface proteases in the cascade of events leading to metastatic spread of tumors; 4) the control mechanisms involved in the regulation of pigment production in normal and in transformed melanocytes. Results obtained have demonstrated that various murine melanomas elicit tumor rejection; this antigen, termed B700, a glycoprotein of 65 kDa m.w., is related biochemically and immunologically to a normal melanocyte protein, perhaps a differentiation antigen. The S91 melanoma that contains a "unique" TSTA in contrast to all other melanoma antigens under study, although it is immunogenic and produces B700, does not express it on the cell surface and cannot be successfully immunized with antigen. We have found that the immune response elicited by B700 is mediated through cytotoxic antibody production and not through a demonstrable cellular response; production of murine monoclonal cytotoxic antibodies is being performed. The involvement of cell surface urokinase with metastatic potential has been shown by use of specific antibodies which can modulate that potential; we have now isolated melanoma cell clones which vary in their tumorigenic and metastatic properties and are employing transfection protocols with the gbene for murine urokinase to further elucidate the mechanisms involved. We have further characterized the molecular controls involved in mammalian pigmentation and its activation by environmental stimuli employing synthetic oligonucleotide and peptide probes to different pigmentation related gene products. The results have shown that melanin production is regulated primarily through a post-translational activation of latent enzyme and that one or more genes normally expressed by melanocytes are repressed in transformed melanocytess while other melanoma-specific genes are activated following transformation.

Another aspect of our work is a study of the highly conserved class I regulatory element (CRE). To study the site of protein binding in more detail, methylation interference experiments were performed. With this method, binding sites are detectable because methylation of guanine residues interferes with protein binding. For region I, methylation of four consecutive G residues from position -172 to -169 in the coding sequence showed clear interference. In region II, the methylation of the G residues from -200 to -191, and in region III the G residues from -189 to -179 and from -173 to -162 which are both within the inverted repeats

showed interference. These data were in full agreement with results obtained by competition assays performed with a series of mutant oligonucleotides containing a few nucleotide substitutions in each of the three regions. The identification of three independent nuclear factors that bind to overlapping, but distinct regions of the CRE raises an interesting question as to why there exists multiple transacting factors regulating the expression of class I genes. Interferon (IFN) induces transcription of class I genes through the use of an interferon consensus sequence (ICS) that is present in the 5' upstream region (-139 bp to -167 bp) from the RNA start site of the genes. We have studied the binding of nuclear factors to the ICS by using a gel mobility shift assay. Our results indicate that the ICS binds a constitutive nuclear factor present in various cells irrespective of IFN treatment. Within one hour following treatment with IFN α/β, two inducible proteins which differed in the requirement of de novo protein synthesis were induced. Methylation interference analysis and competition experiments showed that both constitutive and inducible factors bind a 10 bp site (GTTTCACTTCT) within the ICS. This sequence is shared by a number of IFN inducible genes and may represent a basic structure needed for IFN mediated transcriptional induction.

We have continued to analyze T cell hybridomas from H-2^d mice that recognize the hen egg white lysozyme (HEL) sequence 107-116 in association with the I-E^d molecule. HEL peptide binding studies have been carried out with I-E^d and I-A^d purified proteins and the results indicate that a series of peptides containing region 105-120 binds I-E^d and not I-A^d. The shortest peptide, residues 107-116, binds to I-E^d about 10-fold better than the other peptides, suggesting that a shorter peptide chain could more easily assume the proper conformation for binding to the I-E^d molecules. Within the 107-116 region an ARG to HIS substitution at position 114 profoundly decreases the binding of the peptide to the I-E^d molecule and prevents T cell activation. This variant was also shown to be nonimmunogenic. Therefore, binding of a peptide to Ia molecule is a prerequisite in determining its immunogenicity. A second study which has now been completed is an analysis of the correlation between the interaction of various synthetic peptides encompassing the entire HEL sequence with I-Aa^k molecules and the T cell response to these peptides. Three immunodominant T cell epitopes were found. Two were recognized in association with I-A^k molecules and another with I-E^k molecules.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03200-19 LCBGY

PERIOD COVERED

October 1, 1987 through September 30, 1988

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.	Research Biologist	LCB, NCI
	S. J. Ullrich	Sr. Staff Fellow	LCB, NCI
	K. Kameyama	Visiting Fellow	LCB, NCI
	W. D. Vieira	Microbiologist	LCB, NCI

COOPERATING UNITS *(if any)*

Pittsburgh Cancer Institute, Pittsburgh, PA
Georgetown University Medical School, Washington, DC

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	4	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.)*

Type I (restricted) and Type II (crossreacting) tumor antigens of the transplantation rejection type (TATA), and of tumor antigens (TA) assayed by other in vivo and in vitro techniques and of the immune responses they evoke have received major emphasis. 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. Purification of TATAs is under investigation with the ultimate purpose defining these membrane and cytosol antigens, after purification, in physicochemical, biologic and molecular terms and of assessing their diagnostic and therapeutic potentials.

ANALYSIS OF TSTA OF MCA-INDUCED SARCOMAS

The gp96, p82 and p86/84 antigens.

We have initiated a collaborative study (with A. B. DeLeo, Pittsburgh Cancer Institute) of characterizing a newly isolated, individually distinct TATA of Meth A and other carcinogen induced sarcomas. This gp96 component, isolated and purified from cell membranes, is retained by lentil Lectin columns and is quite distinct from the 82 and 86/84 protein antigens primarily purified in this laboratory. gp96 has now been chemically purified using ion exchange chromatography and specific activity is restricted to peak 2A of a mono Q column (HPLC). gp96 has also been isolated from the CII-7 and CMS-5 sarcomas. Tumor immunity elicited by purified antigen is tumor specific and dose related in the range from 5-25μg; higher concentrations of antigens are yet available for further assays. We are now pursuing amino terminal sequencing and cloning of the gene for gp96 from Meth A and other tumors and BALB/c normal tissues in order to define, in structural forms, specific immunogenicities and polymorphism and comparison with the p82, p86/84 TSTA.

Studies performed in the Chemistry Section have further characterized p82 and the p86/84 cytosolic proteins from Meth A (also isolated from other MCA-induced sarcomas). The p84 isoform has been sequenced and appears to map to chromosome 17 of the mouse. Full length cDNA has also been sequenced for the 86 kD; p84 and p86 are 80% homologous. DNA sequences related to p86 have been assigned to chromosome 12, 11 and 3.

MURINE MELANOMA ANTIGENS (TSTA)

A. Characteristics of Melanoma Antigens

The melanoma TSTA we have studied in this laboratory (with Hearing) differ from most other described TSTA in that they: 1) show extensive crossreactivity (K1735, JB/RH, JB/MS and B16)---melanomas of different etiology and strain of mouse, 2) but also show tumor specificity; S91 immunizes against S91 but not against other melanomas; that is it is a "unique" TSTA, 3) all of the TSTA, unique and crossreacting, are strictly melanoma-specific and are not found on normal tissues, 4) a soluble TSTA termed B700 that is a glycoprotein of 65 kD is capable of immunizing in a fashion identical of the crossreactivity described above with intact cells. This 65 kD is now being used to produce mouse Mabs, 5) immunization with melanoma cells or soluble gp65 provides a striking inhibition of lung metastases; this indicates a T cell immune response. In addition, however, we have now observed a humoral response to the gp65 antigen. These results are described below.

B. Serologic Analysis of Melanoma Antigens

In collaboration with DeLeo (Pittsburgh Cancer Center), analyses of the shared and unique melanoma antigens have been accomplished serologically. Syngeneic antisera against JB/RH (shared) and S91 (unique) were produced by a method used for the preparation of Meth A antisera. Cytotoxic antisera raised against JB/RH were positive for all melanomas expressing the common TSTA but not against S91 and S91 cytotoxic antisera were positive only for S91; specificity was also obtained by quantitative absorptions with intact cells and with the purified B700 melanoma antigen. Thus antibody response may be the major immunologic response against melanoma antigens. This appears to be borne out by results of recent experiments in which two different melanoma-specific monoclonal antibodies (raised against B16 melanoma cells and purified melanoma antigen B700, respectively) were effective in strikingly inhibiting the growth of already established metastases in our JB/MS melanoma model. This specific mechanisms involved in serum inhibition of melanotic growth are under study.

THE JB/MS MELANOMA MODEL AND LUNG METASTASES

In addition to the studies referred to by Hearing on the influence of surface urokinase activity on the metastatic behavior of melanoma cells, we are continuing studies (with C-C. Ting) on the effects, individually and in combination, of indomethacin, interleukin 2 (IL-2) and lymphokine-activated (LAK) cells on established lung metastases using the recently derived, highly immunogenic melanoma JB/MS. Preliminary results indicate that JB/MS is more resistant than the commonly used B16 melanoma to biologic response modifiers. We report the following preliminary results:

1) IL-2 was effective in inhibiting the growth of established (3 day) lung metastases when given every 8 hours or daily but only at a high dose of 8×10^3 units and when begun at day 3. Concentrations as low as 10^2 units were ineffective

2) Chronic indomethacin therapy had no influence on lung metastases when given alone or in combination with IL-2, in contrast to the striking inhibition reported in the B16 model

3) LAK cells or T3 LAK cells (cells activated with anti-CD3 Mab) had no influence on the growth of established JB/MS metastases when transferred adoptively on a single day or on multiple days. Some influence, but sporadic, was seen in combination of IL-2 and LAK cells administered on day 7

4) Daily injections of the effective dose of IL-2 (8×10^3 units) had no effect on metastases in C57Bl mice previously immunized against JB/MS.

The characteristics of the killer cells from spleen and lymph nodes in these several therapeutic trials are being studied in vitro.

PUBLICATIONS

Law LW, Vieira WD. Kameyama K, Hearing VJ. A "unique" tumor-rejection antigen (TSTA) from the S91 murine malignant melanoma. Cancer Res 1987;47:5841-5.

Hearing VJ, Cannon GB. Vieira WD, Jimenez-Atienzar M, Kameyama K, Law LW. JB/MS murine melanoma: A new model for studies of modulation of differentiation and of tumorigenic and metastatic potential. Int J Cancer 1988;41:275-82.

Hearing VJ, Law LW, Corti A, Appella E, Blasi F. Cell surface urokinase modulates the metastatic potential of B16 melanoma cells. Cancer Res 1988;48:1270-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03229-19 LCBGY

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 chererters 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, laboretory, and institute affiliation)*

PI:	E. Appella	Medical Officer (Res.)	LCB, NCI
Other:	S. K. Moore	Sr. Staff Fellow	LCB, NCI
	J. W. Romano	IRTA Fellow	LCB, NCI
	E. A. Robinson	Chemist	LCB, NCI
	S. J. Ullrich	Sr. Staff Fellow	LCB, NCI
	K. Ozato	Res. Microbiologist	LDMI, NICHD
	E. D. Korn	Chief, Lab. Cell Biol.	LCB, NHLBI

COOPERATING UNITS *(if any)*

Weizmann Institute of Sciences, Rehovot, Israel
Preclinical Research, Sandoz Ltd., Basel, Switzerland
Mikrobiologisk Inst., Copenhagen, Denmark

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Chemistry

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	5	1

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.)*

Our research involves the molecular structure of histocompatibility antigens, T cell helper and suppressor receptors and tumor antigens using a combination of protein and DNA sequencing in conjunction with peptide and nucleotide synthesis. Recombinant DNA constructs were used to elucidate the contribution of individual amino acids as well as of the individual $\alpha 1/\alpha 2$ domains of class I antigens toward allo and H-2 restricted CTL reactivity. We have identified three distinct factors in nuclear extracts which binds discrete sequences within the H-2 class I regulator element. Our data also indicate that in the interferon consensus sequence of class I genes, a 10 bp site binds both constitutive and inducible factors.

One of the tumor specific transplantation antigens of the Meth A tumor has been shown to consist of two protein isoforms identified as being the 90 kDa heat shock proteins (hsp). The DNA sequence of these two hsp from Meth A has been completed and that of their normal counterparts is currently being pursued. Chromosomal assignment and localization of one isoform has been found to be near the MHC locus on chromosome 17. Complete sequence analysis of these antigens and their normal counterparts should permit identification of the molecular changes which elicit cell mediated immunity.

DNA sequences predicted to have aberrant helices d(CGCAGAATTCGCG) and d(CGCGAAATTTACGCG) have been crystallized. These sequences showed an overall B-type conformation with two unpaired adenosines looped out from the double helix. These structures are important for defining mechanisms of mutations.

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

HISTOCOMPATIBILITY ANTIGENS

Several studies have approached the question of the recognition of class I histocompatibility antigens by T lymphocytes using products of class I genes derived from in vitro recombinants. Previously we studied a set of exon shuffled class I molecules produced by recombination between H-2D^p and H-2D^d genes. Comparison of these exon shuffled genes with those of H-2D^p and H-2D^d allowed us to implicate particular amino acid residues in the formation of specific serological epitopes. We have now extended our studies of the recognition of these genes by cytolytic T cells (CTLs). Both polyclonal allo and H-2 restricted CTLs require that α1 and α2 protein coding domains be derived from the same haploytpe. As in the case of monoclonal antibodies, we have shown that there are certain T cells which can recognize either α1 or α2 alone. Of further interest is the apparently more rigorous restriction on LCMV specific CTLs to recognize both α1 and α2 of H-2D^b. This indicates that the LCMV peptide binding site formed by a combination of α1 and α2 domains might be more sensitive to neighboring residues than allogeneic recognition.

A second aspect of our work is a study of the highly conserved class I regulatory element (CRE) which is involved in the regulation of transcription of class I genes. We have identified, in nuclear extracts, three distinct factors binding to regions I, II and III within the CRE. To study the site of protein binding in more detail, methylation interference experiments were performed. With this method, binding sites are detectable because methylation of guanine residues interferes with protein binding. For region I, methylation of four consecutive G residues from position -172 to -169 in the coding sequence showed clear interference. In region II, the methylation of the G residues from -200 to -191 and in region III the G residues from -189 to -179 and from -173 to -162 which are both within the inverted repeats showed interference. These data were in full agreement with results obtained by competition assays performed with a series of mutant oligonucleotides containing a few nucleotide substitutions in each of the three regions. The identification of three independent nuclear factors that bind to overlapping, but distinct, regions of the CRE raises an interesting question as to why there exists multiple transacting factors regulating the expression of class I genes. Interferon (IFN) induces transcription of class I genes through the use of an interferon consensus sequence (ICS) that is present in the 5' upstream region (-139 bp to -167 bp) from the RNA start site of the genes. We have studied the binding of nuclear factors to the ICS by using a gel mobility shift assay. Our results indicate that the ICS binds a constitutive nuclear factor present in various cells irrespective of IFN treatment. Within one hour following treatment with IFN α/β, two inducible proteins which differed in the requirement of de novo protein synthesis were induced. Methylation interference analysis and competition experiments showed that both constitutive and inducible factors bind a 10 bp site (GTTTCACTTCT) within the ICS. This sequence is shared by a number of IFN inducible genes and may represent a basic structure needed for IFN mediated transcriptional induction.

T CELL ANTIGEN RECOGNITION

We have continued to analyze T cell hybridomas from H-2^d mice that recognize the hen egg white lysozyme (HEL) sequence 107-116 in association with the I-E^d molecule. HEL peptide binding studies have been carried out with I-E^d and I-A^d purified proteins and the results indicate that a series of peptides containing region 105-120 binds I-E^d and not I-A^d. The shortest peptide, residues 107-116, binds to I-

E^d about 10-fold better than the other peptides, suggesting that a shorter peptide chain could more easily assume the proper conformation for binding to the I-E^d molecules. Within the 107-116 region an ARG to HIS substitution at position 114 profoundly decreases the binding of the peptide to the I-E^d molecule and prevents T cell activation. This variant was also shown to be nonimmunogenic. Therefore, binding of a peptide to Ia molecule is a prerequisite in determining its immunogenicity. A second study which has now been completed is an analysis of the correlation between the interaction of various synthetic peptides encompassing the entire HEL sequence with I-A^k molecules and the T cell response to these peptides. Three immunodominant T cell epitopes were found. Two were recognized in association with I-A^k molecules and another with I-E^k molecules. Several other HEL peptides although capable of binding the appropriate Ia molecules, were not immunodominant. The absence of immunodominance could be shown to arise by 1) competition by an immunodominant peptide for presentation in vivo 2) failure to generate the peptide during antigen processing 3) poor response of the T cell repetoire to a particular peptide MHC complex.

A series of T helper cell hybridomas have been developed from several mouse strains which are restricted by various class II haplotypes and are specific for different peptides of HEL. These hybridomas provide a valuable resource for studying the genetics involved in specific Ag-MHC complex recognition by the T cell receptor (Tcr). Consequently a cDNA library from hybridoma 1H11 (B10.02 derived, I-E^d restricted, HEL peptide 105-120 specific) has been developed. The Tcr beta chain gene has been isolated from this library, sequenced and identified as the Vβ8.2 gene rearranged to the J_β sequence of V_β 1.9.2. A number of Tcr alpha chain clones have been isolated and are currently being characterized. The overall goal is to obtain DNA sequence for the alpha and beta chain genes from a number of these hybridomas and analyze these sequences for similarities and differences which could be correlated to their Ag-MHC specificities.

PROTEIN AND NUCLEIC ACID CHEMISTRY

In recent years several studies have indicated that human lymphocytes are able to produce factors, activating neutrophiles or monocytes chemotaxis, when stimulated with mitogens. We have carried out a detailed biochemical and chemical analysis of these cytokines. Two proteins of about 72 amino acids have been purified and their primary structure determined. These molecules have sequence similarity (up to 45%) with different molecules such as β-thromboglobin, platelet factor 4 and γIP-10, an interferon-induced product of U937 monocyte-like cell line. All of these products constitute a new family of proteins involved both in inflammation and immune regulation.

The oligonucleotide d(CGCAGAATTCGCG) has been crystallized in the conformation of a B-DNA duplex with two unpaired adenosines looped out from the double helix with little perturbation of the rest of the structure. This structure is important because it may give a clue to why certain sequences have a greater tendency to undergo frameshift mutations. A correlation of the observed frequencies of frameshift mutations in various DNA sequences with possible differences in the way the additional nucleotides are accommodated is now being investigated.

Another nucleotide d(CGCGAAATTTACGCG) containing two impaired adenine bases has been crystallized and its structure based on 3Å X-ray diffraction data has been solved. Our data indicate that this oligonucleotide formed a slightly distorted B-type helix with the two unpaired adenine residues looped out. The sequence of

the above oligonucleotide contains a hot spot for purine deletion and its structure promotes our understanding of the mechanisms of mutagenesis. During biosynthesis, if there is a stronger interaction between the adenine and the polymerase than between the adenine and its flanking bases, then the complementary thymidine on the opposite strand may not be incorporated and a single base deletion mutation will be formed.

ANALYSIS OF TUMOR-SPECIFIC TRANSPLANTATION ANTIGENS

There have been three tumor-specific transplantation antigens (TSTA) purified from the Meth A tumor. Our laboratory has described two of these antigens, one of which has been shown to be a major cytosolic heat shock protein (hsp) consisting of two isoforms of 84 and 86 kDa and the other a less abundant cytosolic 82 kDa protein also present in nontransformed cells. Recently, Srivastava et al have purified a third TSTA of 96 kDa and have isolated a corresponding partial genomic clone, but they were unable to identify it as a previously defined protein. We have now identified the nature of this protein, using a synthetic peptide antisera against the 96 kDa antigen, as the 97 kDa glucose regulated protein (GRP), a member of the 84 and 86 kDa hsp family. All of the Meth A TSTAs thus have normal counterparts in nontransformed cells which are indistinguishable by molecular mass, isoelectric point or by the individual anti-TSTA sera from the corresponding Meth A TSTA.

The genes coding for both the 84 and 86 kDa hsp-related TSTAs have been isolated from Meth A tumors. A full length cDNA for the Meth A 84 kDa hsp was previously sequenced; it is an acidic protein of 724 amino acid residues. DNA sequences homologous to hsp 84 were previously mapped to three different chromosomes: 17, 12 and 2. Using specific oligonucleotide probes, the active gene was mapped to chromosome 17. The chromosomal location of hsp 84 relative to the MHC class I region is currently being mapped. The chromosome 17 hsp 84 gene from nontransformed murine cells is now complete in the protein coding region;, no differences between it and the Meth A hsp 84 were found in this region. The gene is located on about 6 kb of DNA and is interrupted by 10 introns. Full length cDNA has also been sequenced for the 86 kDa hsp from Meth A tumors; it is an acidic protein of 733 amino acid residues. The 84 and 86 kDa hsp isoforms are 80% homologous. The protein coding regions are co-linear except for an insertion of 9 amino acids near the N-terminus of hsp 86. DNA sequences related to hsp 86 have been assigned to mouse chromosomes 12, 11 and 3. The gene coding for hsp 86 from normal mouse cells is currently being isolated. Comparison of the normal and Meth A sequences is expected to reveal any changes which may have occurred between this TSTA and its normal cellular counterpart.

Western blot analysis was performed on in vivo and in vitro grown Meth A cells and compared to in vitro NIH 3T3 cultures using polyclonal antisera raised against the various TSTAs. The steady state level of the 82 kDa antigen was elevated approximately 10-fold in either in vivo or in vitro grown Meth A cells compared to its normal counterpart in NIH 3T3 cultures, whereas the in vitro steady state levels of the hsp 84/86 and the 96 kDa TSTAs in Meth A cultures were the same as their normal counterparts in NIH 3T3 cells. However, in vivo Meth A cells displayed lower levels of the 84 kDa hsp isofrom and increased amount of the nonglycosylated form of GRP 97 compared to in vitro cultured Meth A or NIH 3T3 cells; these differences could be reversed by 24 h of in vitro culture. The decreased expression of the 84 kDa TSTA was apparently not solely due to elevated levels of interferon gamma in

vivo, because long term treatment of in vitro cultures with interferon gamma had no effect on the expression of the 84 or 86 kDa antigens.

We have previously shown by immunofluorescence and immune electron microscopy, using affinity purified antisera, that the 82 kDa and the hsp related TSTAs are present in the cytoplasm and at the cell surfacea of in vitro grown cell cultures. However, these experiments did not indicate whether or not these antigens are present at the cell surface as intact molecules or partially degraded polypeptides or the mechanism whereby intracellular proteins are expressed at the cell surface. In order to define the molecule weight of the antigens present at the cell surface of Meth A cells and to extend these observations to Meth A tumors in vivo, the expression of these antigens was examined using the ^{125}I/lactoperoxidase technique to radiolabel cell surface molecules of tumor cells grown in vitro and in vivo. All three Meth A TSTAs could be immunoprecipitated from cell surface labeled in vivo or in vitro lysates using the individual anti-TSTA sera. The level of the 82 kDa TSTA and the 84 and 86 kDa TSTAs was increased approximately 2-fold in in vivo compared to in vitro Meth A cells. The level of the 96 kDa TSTA was essentially unchanged between in vivo and in vitro cells. However, the 95 kDa unglysosylated form of GRP 97 is unable to be iodinated by the lactoperoxidase protocol, and thus cannot be quantitated. In addition to intact hsp 84 and 86, the anti-hsp sera immunoprecipitated several lower molecular weight species ranging from 75 to 35 kDa; these apparently represent degradation products of the 84 and 86 kDa TSTAs. The radiolabeled lysates were also tested for the presence of another intracellular protein, hsp 70, and, as was found for the 82 kDa and the 84/86 kDa TSTAs, a 2-fold increase was seen in in vivo cell lysates. Furthermore, comparison of 2D gel patterns of cell-surface iodinated lysates to ^{35}S-methionine-labeled lysates indicated that other normally intracellular proteins such as actin and GRP 78 were also present at the cell surface of Meth A cells. These data along with the immunofluorescence and E.M. results suggest that a likely mechanism for the presence of the cytosolic 82 kDa and the 84/86 kDa TSTAs at the cell surface is due to cell lysis followed by binding of these relatively abundant proteins to the cell surface. The increased amount of these proteins found in vivo compared to in vitro cells is probably due to increased cell death due to low levels of glucose in vivo and/or specific or nonspecific T cell lysis. Finally the fact that two of the Meth A TSTAs are heat shock-related proteins may have important implications in the mechanisms responsible for generating the antigenicity of the TSTAs and in the immune response to these antigens.

PUBLICATIONS

Hospattankar AV, Fairwell T, Appella E, Meng M, Brewer HB Jr. Human proapolipoprotein A-I: Development of an antibody to the propeptide as a probe of apolipoprotein A-I biosynthesis and processing. Biochem Biophys Res Comm 1987;149:289-96.

Yoshimura T, Matsushima K, Tanaka S, Robinson EA, Appella E, Oppenheim JJ Leonard EJ. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. Proc Natl Acad Sci USA 1987;84:9233-7.

Roy S, Sklenar V, Appella E, Cohen JS. Conformational perturbation due to an extra adenosine in a self-complementary oligodeoxynucleotide duplex. Biopolymers 1987; 26:2041-52.

Sugita K, Miyazaki J, Appella E, Ozato K. Interferons increase transcription of a major histocompatibility class I gene via 5' interferon consensus sequence. Mol Cell Biol 1987;7:2625-30.

Koeller D, Lieberman R, Miyazaki J, Appella E, Ozato K, Mann DW, Forman J. Introduction of H-2^d determinants into the H-$2L^d$ antigen by site directed mutagenesis. J Exp Med 1987;166;744-60.

Moore SK, Kozak C, Robinson EA, Ullrich SJ, Appella E. Cloning and nucleotide sequence of the murine hsp84 cDNA and chromosome assignment of related sequences. Gene 1987;56:29-40.

Appella E, Weber IT, Blasi F. Structure and function of epidermal growth factor-like regions in proteins. FEB Ltrs 1988;2041-52.

Kobayashi Y, Appella E, Yamada M, Copeland TD, Oppenhein JJ, Matsushima K. Phosphorylation of intracellular precursors of Human IL-1. J Immunol 1988;140: 2279-87.

Adorini L, Sette A, Buus S, Grey HM, Darsley M, Lehmann PV, Doria G, Nagy ZA, Appella E. Interaction of an immunodominant epitope with Ia molecules in T-cell activation. Proc Natl Acad Sci USA (in press).

Kanda T, Macchi MJ, LaPan K, Darsley M, Appella E, Frelinger JA. Cytotoxic T lymphocyte recognition of hybrid MHC class I molecules. J Immunogenet (in press).

Matsushima K, Morishita K, Yoshimura T, Lavu S, Kobayashi Y, Lew W, Appella E, Kung H-F, Leonard EJ, Oppenheim JJ. Molecular cloning of a human monocyte derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleuking 1 and tumor necrosis factor. J Exp Med (in press).

Atkinson MAL, Appella E, Corigliano-Murphy M, Korn ED. Enzymatic activity and filament assembly of acanthamoeba myosin II are regulated by adjacent domains at the end of the tail. FEBS Ltrs (in press).

Miller M, Harrison RW, Wlodawer A, Appella E, Sussman JL. Crystal structure of 15-mer DNA duplex containing unpaired bases. Nature (in press).

Joshua-Tor L, Rabinovich D, Hope H, Frolow F, Appella E, Sussman JL. Nature (in press).

Ullrich SJ, Appella E, Mercer WE. Growth related expression of a 72,000 molecular weight poly (A)+ mRNA binding protein. Exp Cell Res (in press).

Tanaka S, Robinson EA, Appella E, Miller M, Ammon HL, Weber IT, Wlodawer A. Structures of amidohydrolases: Amino acid sequence of a glutaminase-asparaginase from Acinetobacter glutaminasificans and preliminary crystallographic data for an asparaginase from Erwinia chrysanthemi. J Biol Chem (in press).

Shirayoshi Y, Burke PA, Appella E, and Ozato K. Interferon-induced transcription of major histocompatibility class I gene accompanies binding of inducible nuclear factors to the interferon consensus sequence. 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 09100-5 LCBGY

PERIOD COVERED

October 1, 1987 through September 30, 1988

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
	Mercedes Jimenez-Atienzar	Visiting Associate	LCB, NCI
	Koichiro Kameyama	Visiting Fellow	LCB, NCI

COOPERATING UNITS *(if any)*

Mikrobiologisk Inst., Copenhagen, DK --- Pittsburgh Cancer Inst., Pittsburgh, PA
Georgetown University Medical Center, Washington, DC
University of Arizona Health Sciences Center, Tucson, AZ

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Chemistry

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	3	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.)*

This project is aimed at characterizing: (1) the host immune response to malignant melanoma, and the role it plays in the progression of tumor growth and metastatic spread; (2) tumor specific proteins produced by melanoma cells in vivo and in vitro, 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 melanoma; (3) the role of cell surface proteases in the cascade of events leading to metastatic spread of tumors; (4) the control mechanisms involved in the regulation of pigment production in normal and in transformed melanocytes. Our results have demonstrated that various murine melanomas of different origin share a common cell surface antigen which can elicit tumor rejection; this antigen, termed B700, is related biochemically and immunologically to a normal melanocyte protein, perhaps a differentiation antigen. The S91 melanoma, although it is immunogenic and produces B700, does not express it on the cell surface, and can not be successfully immunized with that antigen. We have found that the immune response elicited by B700 is mediated through cytotoxic antibody production, and not through a demonstrable cellular response; production of murine monoclonal cytotoxic antibodies is being performed. The involvement of cell surface urokinase with metastatic potential has been shown by use of specific antibodies which can modulate that potential; we have now isolated melanoma cell clones which vary in their tumorigenic and metastatic properties, and are employing transfection protocols with the gene for murine urokinase to further elucidate the mechanisms involved. We have further characterized the molecular controls involved in mammalian pigmentation and its activation by environmental stimuli, employing synthetic oligonucleotide and peptide probes to different pigmentation related gene products. The results have shown that melanin production is regulated primarily through a post-translational activation of latent enzyme, and that one or more genes normally expressed by melanocytes are repressed in transformed melanocytes, while other melanoma-specific genes are activated following transformation.

IMMUNOGENICITY OF MALIGNANT MELANOMA

Previously, we (with Law) have shown that several murine melanomas, including the K1735, JB/RH, JB/MS, B16 and Harding-Passey, share tumor specific transplantation antigens (TSTA), which do not cross-react with the S91 melanoma, although the latter produces a specific TSTA. The cross-reactive melanoma TSTA is extremely interesting since the tumors expressing it have arisen over a period of decades as the result of spontaneous events or by chemical or ultraviolet induced transformation. We have shown that B700, which is a melanoma tumor specific antigen (TSA, see below), when isolated from the above tumors, can be used to immunize mice and elicit tumor rejection activity, with the specificity noted above. We are engaged in characterizing the mechanisms involved in these responses, and although there is no significant cellular immune response, there is a dramatic humoral response. Our studies (with Law and DeLeo) have shown that mice immunized with melanoma cells produce high titers of cytotoxic antibodies, which can be quantitatively absorbed with the B700 antigen. The specificity of this cytotoxicity is identical to the pattern of cross-reactive TSTA activity noted above, suggesting that the major response to challenge with melanoma cells is the production of cytotoxic antibodies and not the generation of cell mediated immune mechanisms. Since these responses are rarely effective in preventing tumor growth in naive animals, our current studies are directed at examining the reasons behind this ineffective response, and at ways to successfully augment it.

TUMOR SPECIFIC ANTIGENS PRODUCED BY MALIGNANT MELANOMA

Transformed cells express dramatically different cellular products than do their normal counterparts; the production of novel products (TSA) by malignant cells (i.e. those not produced by normal cells) and the involvement of TSA in the growth and function of tumor cells has been a subject of widespread interest. We (with Gersten) have examined two such TSA, termed B700 and B50 - both of these antigens seem to be analogous to normal melanocyte proteins, but with alterations in their primary structure which gives rise to their immunologic specificity. The ability of B700 to also function as a TSTA in cells which express it on the cell membrane surface has recently been shown by our group (see above). We have also found that B700, or a cross-reactive antigen, is expressed specifically by melanomas of human, hamster and swine origin, in addition to the murine melanomas. We are currently in the process of producing murine monoclonal antibodies to B700, and are collaborating on a project to clone its gene; such information should allow us to characterize the mechanism(s) whereby this TSA is produced by transformed melanocytes. We (with Leong) have also examined the specificity of another melanoma specific antigen, termed 1G6, which was identified by syngeneic monoclonal antibodies produced against B16 melanoma cells. Our studies have shown that this antigen is expressed only by melanomas arising in C57Bl mice (either spontaneous or chemically induced); interestingly, expression of the antigen is dependent on the replication and budding of a B-tropic ecotropic murine retrovirus. Whether the antigen is encoded by the virus, or merely induced to express specifically by melanocytes producing the virus, is currently being studied; the specificity of this antigen with C57Bl derived melanomas is an interesting phenomenon which is also under investigation.

METASTATIC EVENTS IN DISSEMINATION OF MALIGNANT MELANOMA

We (with Appella, Blasi and Law) have examined the role of cell surface membrane expression of proteases on tumor invasion and metastasis. We have shown that urokinase, a protease involved in the control of migration and differentiation of normal cells, is critical to the tumor cell's capability to penetrate membranes and other proteinaceous barriers during metastasis of transformed cells. We have shown that alteration of surface urokinase activity by immunological and enzymatic reagents has dramatic consequences on the metastatic behavior of melanoma cells, which correlates directly with increases or decreases in urokinase catalytic activity. We have addressed the question of whether this parameter is important in vivo by cloning B16 and JB/MS murine melanoma cells, using no selection pressure, then examining the metastatic potentials and the urokinase expression of the cloned lines; again, there was a correlation between metastatic activity and surface localization of urokinase activity. These studies suggest that urokinase is an important, although not a sufficient, parameter in the determination of a tumor cell to metastasize. Our current research is directed at inserting the urokinase gene into poorly metastatic cells in order to directly determine the effect of enhanced urokinase expression on metastatic behavior. In addition, in recognition of the importance of the surface receptor for urokinase, we are attempting to identify and characterize this molecule; future studies will be directed at examining the importance of urokinase receptor expression on metastatic ability.

REGULATION OF MELANOGENESIS

We (with Jiménez and Kameyama) have examined the levels of response of mammalian melanocytes to physiologically relevant stimuli, such as UV light and melanocyte stimulating hormone (MSH). The melanocyte provides a unique model system to study intracellular control mechanisms since only the enzyme tyrosinase is essential for melanin biosynthesis, although the rates of pigmentation can be influenced at various levels. We have produced tyrosinase specific monoclonal antibodies which cross-react among mammalian tyrosinases, and have been valuable probes for the characterization of post-translational enzyme processing in melanocytes. We have used them to examine the metabolic processing of tyrosinase, which includes glycosylation and intracellular transport to melanosomes, where melanization is initiated. There have recently been two different genes cloned which may encode tyrosinase, although no primary sequence is available to confirm such an identification. As an approach to unraveling the gene products involved in the regulation of melanogenesis, we have synthesized oligonucleotides encoded by those genes for hybridization studies, and peptides used to produce specific antibodies, and have examined their expression at the transcriptional and translational level in response to environmental stimuli. In agreement with our earlier studies, we have found only slight increases in the synthesis of de novo tyrosinase in response to melanogenic stimulation, and that the dramatic increases in pigmentation result from activation of latent tyrosinase in the melanocyte. We have now cloned a melanoma cell line that produces a large quantity of a melanogenic inhibitor which may be critical to this masking of catalytic activity and are in the process of purifying and characterizing it. In addition, we have shown that levels of melanogenesis correlate closely with the expression of surface MSH receptors, which in turn can be regulated by various environmental stimuli, such as cytokines (e.g. interferon), ultraviolet light and by MSH itself (as an autocrine function). The emerging picture indicates that the control of mammalian pigmentation is an order

of magnitude more complex than originally thought, but recent advances at the molecular level are slowly unraveling these interrelated control mechanisms, which malfunction in such pigmentary disorders as albinism.

PUBLICATIONS

Law LW, Vieira, WD, Kameyama K, Hearing VJ Jr. A "unique" tumor-rejection antigen (TSTA) from the S91 murine malignant melanoma. Cancer Res 1987;47:5841-5.

Hearing VJ, Jiménez M. Mammalian tyrosinase - the critical regulatory control point in melanocyte pigmentation. Int J Biochem 1987;19:1141-7.

Gersten DM, Phillips TM, Hearing VJ Jr. Temporal synthesis and presentation of antigens by cultured B16 melanoma cells. Pigment Cell Res 1987;1:118-23.

Hearing VJ Jr, Cannon GB, Vieira WD, Jiménez M, Kameyama K, Law LW. JB/MS murine melanoma - a new model for studies on the modulation of differentiation and of tumorigenic and metastatic potential. Int J Cancer 1988;41:275-82.

Hearing VJ Jr, Law LW, Nolli ML, Appella E, Blasi F. Modulation of metastatic potential by cell surface urokinase of murine melanoma cells. Cancer Res 1988:48: 1270-8.

Jiménez M, Kameyama K, Maloy WL, Tomita Y, Hearing VJ Jr. Mammalian tyrosinase: biosynthesis, processing and modulation by melanocyte stimulating hormone. Proc. Natl Acad Sci USA 1988;85:3830-4.

Gersten DM, Hearing VJ Jr. Demonstration of B700 cross-reactive antigens on human and other animal melanomas. Pigment Cell Res (in press).

Kraemer KH, Herlyn M, Yuspa S, Clark WH Jr, Townsend K, Neises G, Hearing VJ, Jr. Reduced DNA repair in cultured melanocytes and nevus cells from a patient with Xeroderma Pigmentosum. Arch Dermatol (in press).

Jacobsohn GM, Chiartas PL, Hearing VJ, Jacobsohn MK. Role of estradiol and 2-hydroxyestradiol in melanin formation. Biochim Biophys Acta (in press).

Leong SPL, Muller J, Yetter R, Gorelik E, Hearing VJ Jr. Expression and modulation of a retrovirally encoded antigen by B16 murine melanomas. Cancer Res (in press).

Kameyama K, Montague PM, Hearing VJ Jr. The expression of melanocyte stimulating hormone receptors correlates with mammalian pigmentation and can be modulated by interferons. J Cell Physiol (in press).

Kameyama K, Tanaka S, Ishida Y, Hearing, VJ Jr. Murine α, β and τ-interferons stimulate melanogenesis by increasing the number of α-melanocyte stimulating hormone receptors on JB/MS murine melanoma cells. J Clin Res (in press).

Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1987 to September 30, 1988

During the last year the three program areas of the Laboratory of Immunobiology have made major advances in their respective fields. In the program of the Cellular Immunity Section analysis of the genetic basis of human kidney and lung carcinomas continued with considerable success; in the Immunopathology Section two new lymphokines have been discovered; and in the Office of the Chief major advances have been made in understanding the molecular basis of activation of complement.

In the Office of the Chief, efforts continued to elucidate the mechanism whereby the classical complement cascade is initiated. Utilizing techniques devised during the last two years in this laboratory we have continued investigating the subcomponents of C1, the first component of the classical complement cascade. C1 is composed of three subcomponents: C1q (the recognition component), C1r (the internal activating proenzyme) and C1s (the proenzyme for activating the C cascade). In the activating sequence, C1q activates C1r which in turn activates C1s. Until we introduced the techniques to investigate native C1 structure-function relations, investigators using purified, labelled C1 subcomponents thought that C1 activation was spontaneous, autocatalytic, controlled by an inhibitor, and antibodies only accelerated this process. We showed last year that this was erroneus for C1 and C1s. We now found the C1r activation is similar to that of C1s. Spontaneous activation of C1r was examined by immunoblotting techniques. We used partially purified C1r isolated by ultracentrifugation from fresh human serum. C1 inhibitor was less than 0.5% of serum. We found that the rate of spontaneous activation was slow (less than 10% in 30 min) and that it was concentration dependent; the rate was enhanced by the addition of already activated C1r and was inhibited by serine protease inhibitors. These results were almost identical with those obtained with C1s and suggested that they were inconsistent with a spontaneous intramolecular autoactivation model and suggested intermolecular activation by contaminating serine proteases or by activated C1r.

Since the picture that emerged from these studies was consistent with our hypothesis of 20 year's standing that activation of C1 was the result of the interaction of C1q with activating substances (such as antigen-antibody complexes) we also continued our studies of C1q structure as relates to function. About 20 years ago we postulated that the deviation of native C1 dose-response curve from linearity was due to concentration dependent dissociation of C1q into smaller fragments, only a fraction of which was associated with C1r and C1s in activable form. Several years ago Dr. M. Loos of Mainz, FRG, a major collaborator of this Laboratory, reported experiments supporting this hypothesis. Dr. Loos' results were never reproduced and were not generally accepted. We have now reinvestigated this question with modern tools. Dissociation of C1q in native form upon dilution was examined by using ultracentrifugation in sucrose gradient and

HPLC using a size exclusion column for separating dissociated fractions. Antigenic content of C1q in each fraction was detected and quantitated by ELISA and by immunoblotting; binding to antibody coated red cells was also determined. The results showed that C1q did dissociate as a function of concentration and that up to 14% of the C1q was in the low MW form. In addition the low MW form could bind to antibody coated red cells. We thus confirmed and extended Loos' observation and strengthened the theoretical basis for non-linearity of native C1 dose-response relationship.

A major effort of the Immunopathology Section during the past year has been characterization of an attractant for human neutrophils that is produced by LPS-stimulated human blood mononuclear cells. This attractant is called monocyte-derived neutrophil chemotactic factor (MDNCF). We first showed that the chemotactic activity in culture fluid of LPS-stimulated mononuclear cells, which was previously attributed to IL-1, is due to a peptide distinct from IL-1. This peptide was then purified to homogeneity from 3 liters of culture fluid in a sequence that included anion exchange, gel filtration, HPLC on a cation exchanger, and finally reverse phase HPLC. The final product showed one band by silver stain on SDS-PAGE. The optimal concentration of MDNCF for neutrophil chemotaxis was 10^{-8}M. Efficacy was 54%, comparable to that obtained with a reference attractant for the formyl-methionyl receptor. Of great interest is the observation that NAP-1 did not attract human monocytes.

In collaboration with Dr. Ettore Appella and Dr. Kouji Matsushima, the amino acid composition and N-terminal sequence of MDNCF was determined; and a probe was constructed that was used to detect MDNCF message in a cDNA library of LPS-stimulated human monocytes. MDNCF has sequence similarity to several other peptides that are released in response to tissue injury. Of particular interest is the similarity to PF-4 and CTAP-III, two peptides released from platelets in response to aggregation or stimulation. PF-4 is a chemoattractant and CTAP-III contributes to tissue repair by stimulating fibroblasts to replicate and secrete glycosaminoglycans. Thus platelet and macrophage products contribute to three key areas of host defense in response to injury -- hemostasis, leukocyte recruitment, and tissue repair.

In order to determine if MDNCF causes neutrophil accumulation in inflammatory exudates, an ELISA assay has been developed, based on the use of two different antibodies--a mouse monoclonal anti-MDNCF and a rabbit polyclonal anti-MDNCF. Detection of MDNCF in biological fluids requires a sandwich assay, in which an anti-MDNCF antibody is bound to the assay plate. This is followed by sequential addition of fluid containing MDNCF and then a second antibody to MDNCF. The assay can detect 1 ng of MDNCF. Cross-reactivity studies show that monoclonal anti-MDNCF also interacts with CTAP-III. However, rabbit anti-MDNCF does not react with CTAP-III. Since the ELISA assay requires interaction with both antibodies, CTAP-III is not detected.

MDNCF has been labeled with fluorescein, at a level of approximately 1 molecule per molecule of peptide. The labeled peptide has the same chemotactic potency and efficacy as the parent molecule. It will be used for receptor studies and to correlate biological activity and binding

levels for different types of leukocytes. Preliminary flow cytometry data show that fluoresceinated MDNCF binds to human neutrophils at levels comparable to that of a fluorescein-labeled ligand for the formyl-methionyl chemotaxis receptor. There is no detectable binding to lymphocytes and only a very low level binding to monocytes, consistent with the unresponsiveness of monocytes to MDNCF.

The culture fluid of LPS-stimulated human blood mononuclear cells also contains a chemotactic activity that attracts monocytes but not neutrophils. The cellular source of this activity is not known at the present time. It may be the activity previously reported to be produced by lymphocytes in response to antigen or mitogen stimulation, and thus could be important in delayed hypersensitivity reactions. Purification of this attractant is in progress.

Human macrophage stimulating protein (MSP) is present in human blood plasma and makes mouse macrophages responsive to chemoattractants. The present objective is to obtain an MSP-specific antibody that could be used in studies of cellular origin, tissue distribution, and plasma concentration. Several mouse monoclonal anti-MSP antibodies have been obtained, but all react with several plasma proteins in addition to MSP. An approach to this problem would be to immunize mice with partial sequences of pure MSP. The current purification schema includes application of human plasma to a monoclonal anti-MSP column, acid elution, and chromatography on an HPLC cation exchange column. Although the purification factor at this stage is about $5x10^5$, the product is not yet sufficiently homogeneous for N-terminal amino acid sequencing.

The Cellular Immunity Section has continued studies of human tumor suppressor genes. Previously we discovered that sporadic human renal cell carcinomas and small cell lung carcinomas were characterized by a loss of DNA sequences on the short arm of chromosome 3 (3p). These observations were interpreted to mean that a tumor suppressor gene, the "renal cell carcinoma" gene was located on chromosome 3p. Genes that lead to cancer when the function of both copies have been eliminated by mutation have been referred to as tumor suppressor genes or recessive oncogenes. Analysis of additional samples of renal cell carcinoma and small cell lung carcinoma in this and other laboratories have confirmed the loss of DNA sequences on chromosome 3p. To obtain additional evidence that a gene that predisposes to renal cell carcinoma is located on chromosome 3p, studies were begun of families with inherited renal cell carcinoma. Von Hippel Lindau disease (VHL) is a genetic defect associated with multiple tumors including renal cell carcinoma. The gene that predisposes to VHL is located on chromosome 3p and is linked to c-raf-1 (3p25). Specific loss of alleles from chromosome 3p was detected with polymorphic DNA probes in four renal cell carcinomas from a patient with VHL. Analysis of haplotypes indicated that the loss of chromosome 3p alleles was from the chromosome bearing the wild-type allele of the VHL gene. The loss of the chromosome 3p bearing the VHL wild-type allele from four renal cell carcinomas is consistent with the concept that renal cell carcinoma associated with VHL is produced as a consequence of a recessive oncogene. The VHL gene may be identical to the gene associated with sporadic and familial renal cell carcinoma.

During the past year studies have been initiated to clone the human renal cell carcinoma gene. Five thousand clones that contain unique human DNA fragments were isolated from two chromosome 3 lambda phage libraries. More than 50% of these single copy clones contained gene-associated transcribed sequences. At present 28 new chromosome 3p probes have been isolated.

A method was developed to improve DNA polymorphism analysis by removal of contaminating host cells from human solid tumors. Leukocytes were removed from primary renal cell carcinomas by sequential treatment with a mouse antibody to a human leukocyte antigen, a goat antibody (bound to iron-containing beads) to mouse immunoglobulin and a magnet. Detection of loss of DNA sequences on chromosome 3p was facilitated in DNA extracted from partially-purified primary renal cell carcinoma populations. One primary renal cell carcinoma that did not show chromosome 3p deletion when the crude tumor mixture was analyzed, did show this change after removal of contaminating host leukocytes.

A study was performed to detect chromosomal loss in human adrenal cortical tumors and in benign adrenal cortical and medullary tumors. Benign adrenal tumors from 12 patients and adrenocortical carcinomas from 7 patients were tested for loss of heterozygosity at loci on human chromosomes 3, 11, 13, 15, 16 and 17. Loss of DNA sequences on chromosomes 11 and 17 was frequently detected in adrenocortical carcinomas. These results suggest that a gene(s) located on chromosome 11p may be important in the origin or evolution of adrenocortical carcinoma and raise the possibility that genes located on chromosome 17 may also play a vital role in tumorigenesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08528-12 LIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI-FCRF, NIH, Frederick, MD 21701

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.)*

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. This project has been terminated.

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

Publications:

Zbar B, Tanio Y. Host-clonal interactions in the generation of proviral gene deletion variants. JNCI 1987;79:383-388.

Talmadge C, Tanio Y, Meeker A, Talmadge J, Zbar B. Tumor cells transfected with the neomycin resistance gene (neo) contain unique genetic markers useful for identification of tumor recurrence and metastasis. Invasion and Metastases 1987;7:197-207.

Talmadge JE, Talamdge CB, Zbar B, McEwen R, Meeker AK, Tribble H. In vivo immunologic selection of Class I MHC gene deletion variants from the B16-BL6 melanoma. JNCI 1987;78:1215-1221.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08552-22 LIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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	Office of the Chief	LIB NCI
OTHER:	S. Hosoi	Visiting Fellow	LIB NCI

COOPERATING UNITS *(if any)*

Department of Biochemistry, University of Lausanne
Department of Microbiology, University of Mainz

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, FCRF, Frederick, MD 21701

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.)*

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 (Rev. 1/84) GPO 914-918

Major findings: we continued elucidation of the activation of the first component of complement (C1). Since we showed last year that spontaneous activation of native C1 was slow and most likely due to contaminating proteolytic enzymes we explored further the mechanism of "spontaneous" activation. We also explored that concentration dependent loss of self activability of C1. Regarding "spontaneous" activation we extended the studies to C1r, the subunit of C1 that is activated by C1q and effects the activation of C1s, the enzyme that initiates the classical pathway. C1r activation was studied in the presense of EDTA by Western blot. Partially purified C1r was prepared by ultracentrifugation of fresh serum in 10-30% sucrose gradient. Final concentration of C1r was about 1/6 of that of the original serum. C1 inhibitor was detected by radial diffusion; none was found (limit of detection: less than 0.5% of original serum content). The results demonstrated that "spontaneous" activation of C1r was slow (less than 10% at 30 min at 37°); was concentration dependent; was enhanced by the addition of activated C1r and was completely suppressed by the addition of serine protease inhibitors. The results were interpreted to mean that activation of C1r was dependent on external signals and that the so-called spontanous activation of purified C1r, like that of C1s, is an artifact of purification.

The subcomponent that activates C1r in the C1 molecule is C1q. C1q is bi-functional: it has the recognition site for immunoglobulins in the globulin portion, and the C1r-C1s binding site in the collagen-like portion. We have shown many years ago that binding of C1 by an antibody-antigen complex does not necessarily lead to activation of C1s. It was proposed that C1q existed in several forms, only some of which were competent to activate C1s. It was proposed and supported by evidence that C1q itself could dissociate into smaller subunits and that an intact C1q was necessary for activation of C1s. We have re-examined dissociation of native C1q upon dilution into subunits by using ultracentrifugation in a sucrose gradient and an HPLC system with size exclusion column for separating putative dissociated subunits of C1q. C1q was detected by ELISA and by Western blotting; binding to red cells bearing complement fixing antibodies (EA) was also determined. The results confirmed and extended previous claims that C1q in native C1 dissociated as a function of concentration; up to 14.5% of C1q antigen was in a low MW form (approximate S value: 4 to 5). As expected, C1q from purified commercial preparation also contained C1q antigen in the low MW form. The low MW form could bind to EA. The results strongly supported the idea that native C1 may contain a fraction of C1 that has incomplete C1q molecules, that the size of the fraction depended on the concentration of C1 and that both form could bind to antibodies but that only C1 containing the intact C1q was capable of self-activation.

Publications:

Borsos T, Leonard EJ. The physiologic functions of complement. In: Oppenheim JJ, Shevach E, eds. Immunophysiology. Oxford University Press, 1988.

Borsos T. Introduction to complement. In: Antoni F, Zavodszky P, eds. Immunfolyamatok molekularis tenyezoi. Hung Acad Sciences, 1988.

Glenn G, Szende B, Yano T, Borsos T, Zbar B. Serotheray of cancer: cellular changes in primary rat mammary carcinomas after infusion of syngeneic sera absorbed with protein A-Sepharose. Int J Cancer 1988; in press.

Hosoi S, Borsos T. Activation of human C1r: Western blotting analysis reveals slow and dose dependent activation. J Immunol. 1988; in press.

Kirschfink M, Borsos T. Binding and activation of C4 and C3 on the red cell surface by non-complement enzymes. Molec Immunol 1988;25:505-512.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08575-16 LIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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:	Teizo Yoshimura	Visiting Fellow	LIB NCI

COOPERATING UNITS *(if any)*

	Kouji Matsushima	Laboratory of Immunoregulation, BRMP

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Immunopathology Section

INSTITUTE AND LOCATION

NCI, NIH, FCRF, Frederick, MD 21701

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.)*

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 914-916

Major findings: a major effort during the past year has been characterization of an attractant for human neutrophils that is produced by LPS-stimulated human blood mononuclear cells. This attractant is called monocyte derived neutrophil chemotactic factor (MDNCF). It was first shown that the chemotactic activity in culture fluid of LPS-stimulated mononuclear cells, which was previously attributed to IL-1, is due to a peptide distinct from IL-1. This peptide was then purified to homogeneity from 3 liters of culture fluid in a sequence that included anion exchange, gel filtration, HPLC on a cation exchanger, and finally reverse phase HPLC. The final product showed one band by silver stain on SDS-PAGE. The optimal concentration of MDNCF for neutrophil chemotaxis was 10-8M. Efficacy was 54%, comparable to that obtained with a reference attractant for the formyl-methionyl receptor. Of great interest is the observation that MDNCF did not attract human monocytes.

In collaboration with Dr. Ettore Appella and Dr. Kouji Matsushima, the amino acid composition and N-terminal sequence of MDNCF was determined; and a probe was constructed that was used to detect MDNCF message in a cDNA library of LPS-stimulated human monocytes. MDNCF has sequence similarity to several other peptides that are released in response to tissue injury. Of particular interest is the similarity to PF-4 and CTAP-III, two peptides released from platelets in response to aggregation or stimulation. PF-4 is a chemoattractant and CTAP-III contributes to tissue repair by stimulating fibroblasts to replicate and secrete glycosaminoglycans. Thus platelet and macrophage products contribute to three key areas of host defense in response to injury -- hemostasis, leukocyte recruitment, and tissue repair.

In order to determine if MDNCF causes neutrophil accumulation in inflammatory exudates, an ELISA assay has been developed, based on the use of two different antibodies--a mouse monoclonal anti-MDNCF and a rabbit polyclonal anti-MDNCF. Detection of MDNCF in biological fluids requires a sandwich assay, in which an anti-MDNCF antibody is bound to the assay plate. This is followed by sequential addition of fluid containing MDNCF and then a second antibody to MDNCF. The assay can detect 1 ng of NAP-1. Cross-reactivity studies show that monoclonal anti-MDNCF also interacts with CTAP-III. However, rabbit anti-MDNCF does not react with CTAP-III. Since the ELISA assay requires interaction with both antibodies, CTAP-III is not detected.

MDNCF has been labeled with fluorescein, at a level of approximately 1 molecule per molecule of peptide. The labeled peptide has the same chemotactic potency and efficacy as the parent molecule. It will be used for receptor studies and to correlate biological activity and binding levels for different types of leukocytes. Preliminary flow cytometry data show that fluoresceinated MDNCF binds to human neutrophils at levels comparable to that of a fluorescein-labeled ligand for the formyl-methionyl chemotaxis receptor. There is no detectable binding to lymphocytes. There is low but significant binding to monocytes, despite unresponsiveness of monocytes to MDNCF.

The culture fluid of LPS-stimulated human blood mononuclear cells also contains a chemotactic activity that attracts monocytes but not neutrophils. The cellular source of this activity is not known at the present time. It may be the activity previously reported to be produced by lymphocytes in response to antigen or mitogen stimulation, and thus could be important in delayed hypersensitivity reactions. Purification of this attractant is in progress.

Human macrophage stimulating protein (MSP) is present in human blood plasma and makes mouse macrophages responsive to chemoattractants. The present objective is to obtain an MSP-specific antibody that could be used in studies of cellular origin, tissue distribution, and plasma concentration. Several mouse monoclonal anti-MSP antibodies have been obtained, but all react with several plasma proteins in addition to MSP. An approach to this problem would be to immunize mice with partial sequences of pure MSP. The current purification schema includes application of human plasma to a monoclonal anti-MSP column, acid elution, and chromatography on an HPLC cation exchange column. Although the purification factor at this stage is about 5x105, the product is not yet sufficiently homogeneous for N-terminal amino acid sequencing.

Publications:

Matsushima K, Morishita K, Yoshimura T, Lavu S, Kobayashi Y, Lew W, Appella E, Kung SF, Leonard EJ, Oppenheim JJ. Molecular cloning of cDNA for a human monocyte derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin-1 and tumor necrosis factor. J Exp Med, in press.

Oppenheim JJ, Matsushima K, Yoshimura T, Leonard EJ. The activities of cytokines are pleiotropic and interdependent. Immunology Letters 1987; 16:179-184.

Rot A, Henderson LE, Copeland TD, Leonard EJ. A new series of ligands for the human formyl peptide receptor: tetrapeptides with high chemotactic potency and efficacy. Proc Natl Acad Sci USA 1987;84:7967-7971.

Yoshimura T, Matsushima K, Oppenheim JJ, Leonard EJ. Neutrophil chemotactic factor produced by lipopolysaccharide (LPS) stimulated human blood mononuclear leukocytes. I. Partial characterization and separation from interleukin-1 (IL-1). J Immunol 1987;139:788-793.

Yoshimura T, Matsushima K, Tanaka S, Robinson EA, Appella E, Oppenheim JJ, Leonard EJ. Purification of a human monocyte-derived neutrophil chemotactic factor that shares sequence homology with other host defense cytokines. Proc Natl Acad Sci USA 1987;84:9233-9237.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08577-03 LIB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Restriction fragment length polymorphisms in normal and neoplastic tissues

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:	M. Lerman	Expert	LIB NCI
	H. Brauch	Visiting Fellow	LIB NCI
	S. Hosoi	Visiting Fellow	LIB NCI
	L. Daniel	Biotechnology Fellow	LIB NCI
	G. Glenn	Medical Staff Fellow	LIB NCI

COOPERATING UNITS *(if any)*

M. Linehan, Surgery Branch, DCT NCI; J. Minna, Navy Medical Oncology Unit, NCI; G. Sorenson, Dartmouth Medical School; F. Li, Epidemiology, NCI, Boston; E. Oldfield, Surgical Neurology Branch, NINCDS

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI-FCRF, NIH, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.0	6.0	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.)*

Loss of alleles on the short arm of chromosome 3 was detected in small cell lung carcinoma and hereditary and non hereditary renal cell carcinoma. Studies of familial renal cell carcinoma were consistent with the concept that a gene that predisposes to renal cell carcinoma is located on chromosome 3p. The method for detection of deletions in human solid tumors was improved. A strategy for isolation of the renal cell carcinoma gene was developed and 3000 clones isolated from a chromosome 3 library.

Major findings:

Renal cell carcinoma. We previously reported the loss of alleles at loci on chromosome 3p in sporadic renal cell carcinoma. Over the past year we have confirmed this observation in studies of 13 additional patients with sporadic renal cell carcinomas. These results suggested that there was a gene that predisposed to renal renal cell carcinoma located on the short arm of chromosome 3.

To test this hypothesis studies were performed in a family in which the mother and both her children had renal cell carcinoma. The mother and her son had bilateral renal cell carcinoma; the tumors were detected at an early age. These clinical features were compatable with the inheritance of a gene that predisposed to renal cell carcinoma. Four renal cell carcinomas from the proband were studied by DNA polymorphism analysis. Eaah renal cell carcinoma had lost the same chromosome 3p and this chromosome was inherited from the non affected parent (the father). The loss of identical chromosome 3 (from the non affected parent) in 4 renal cell carcinomas is consistent with the concept that there is a recessive oncogene located on chromosome 3p that predisposes to renal cell carcinoma.

Immunological techniques were used to improve DNA polymorphism analysis of primary renal cell carcinomas. Removal of host leukocytes from disaggregated renal cell carcinomas facilitated detection of chromosome 3p allele loss.

Isolation of the renal cell carcinoma gene. Five thousand single copy probes were isolated from two flow-sorted chromosome 3 lambda phage libraries. The collection of single copy clones was characterized by screening with a "normalized" human cDNA probe prepared from a mixture of several human cDNA libraries and poly A mRNA's. Greater than 50% of these single copy clones reacted as positive suggested that the randomly selected clones contained a high proportion of gene-associated transcribed sequences. Minprep DNA (about 50 ug) from more than 3000 clones was isolated. To date about 1000 of them were digested with Eco RI and characterized by agarose gel electrophoresis. About 75% of these clones contained inserts greater than 500 bp. The inserts were excised from low-geling agarose gels, labeled by random priming and used to probe blots devised to assign regional location to each probe on chromosome 3. Of about 100 inserts tested so far, 25-30% were located on 3p and 70-75% on 3q.

Publications:

Brauch H, Johnson B, Hovis J, Yano T, Gazdar A, Pettengill OS, Graziano S, Sorenson GD, Poiesz BJ, Minna J, Linehan M, Zbar B. Molecular analysis of the short arm of chromosome 3 in small-cell and non-small-cell carcinoma of the lung. New Eng J Med 1987;317:1109-1113.

Zbar B, Brauch H, Talamdge C, Linehan M. Loss of alleles of loci on the short arm of chromosome 3 in renal cell carcinoma. Nature 1988;327:721-724.

Zbar, B. The significance of loss of DNA sequences on the short arm of chromosome 3 in renal cell carcinoma and small cell lung carcinoma. In: Important Advances in Oncology 1989 edited by DeVita, Hellman, Rosenberg, in press.

Zbar, B. Evidence for a recessive oncogene on human chromosome 3: implications for pathologists and clinicians. In: Molecular Genetics and the Diagnosis of Cancer edited by Cossman, in press.

SUMMARY REPORT
IMMUNOLOGY BRANCH
October 1987 - September 1988

The Immunology Branch carries out laboratory investigations in basic immunology with particular emphasis on studies of the major histocompatibility complex (MHC) and its role in transplantation in animal models and in man. The major areas of study are as follows: 1) Immunogenetics of the murine MHC; 2) Development of models for the induction of specific tolerance to MHC products; 3) Genetics of the miniature swine MHC; 4) Molecular biology of porcine class II genes; and 5) Studies of bone marrow and organ transplantation in miniature swine. This report summarizes research efforts in each of these areas during the past year, with reference to the individual annual reports in which details can be found.

1. IMMUNOGENETICS OF THE MURINE MHC

A breeding program is carried out for the maintenance of pedigreed inbred and congenic resistant strains of mice and for the development of new recombinant H-2 haplotypes of value for structural and functional studies of genes within the MHC (5023). This program also includes the production of monoclonal antibodies reactive with products of MHC genes. New strains bearing recombinant MHC haplotypes (of which 22 have been derived from the breeding program of the Immunology Branch) are utilized for the production of new reagents and probes to study the fine structure of the MHC.

One recent recombinant, the C3H.KBR, was derived during the backcrossing of the C3H.SW to C3H. This recombinant strain produced a surprisingly high anti-Qa reactivity when immunized against C3H.SW. A fusion from immunized spleen cells of this strain produced a panel of ten new monoclonal antibodies all reactive with Qa antigens. These new antibodies have been used to define at least three epitope groups on the Qa-2 molecule. Immunoprecipitation studies have indicated that at least two separable molecules can be detected with these antibodies, the first set reactive with the entire panel, and the second set reactive with all but one of the monoclonal antibodies. Functional studies using these monoclonal antibodies have indicated marked differences in their effects in vitro and in vivo. Flow microfluorometry studies indicate that differences in the ability to induce Qa modulation may be the basis for this difference in functional effect. If so, these results may provide a clue to the natural function of ligand interactions with the Qa molecule.

Another new recombinant derived from this program, the A.TH-Q1b, has made possible for the first time an analysis of the A.TH anti-A.TL reactivity in the absence of a Qa-1 difference. Skin grafts between the new A.TH-Q1b animals and ATL were found to be markedly prolonged, indicating that class II differences alone may be insufficient to cause prompt skin graft rejection. These data may clarify the previously puzzling finding that some class II antigens cause skin graft rejection while others do not. The data suggest that one role of class II differences in skin graft rejection may be the presentation of other minor antigenic differences to the immune system.

2. DEVELOPMENT OF MODELS FOR THE INDUCTION OF SPECIFIC TOLERANCE TO MHC PRODUCTS

The induction of intentional mixed chimerism across MHC barriers has been investigated as a means of producing specific transplant tolerance (5021). The use of mixtures of T-cell depleted syngeneic plus allogeneic bone marrow has been found to produce permanent, specific tolerance with full immunocompetence. Reconstitution of irradiated animals with a mixture containing T cell depleted syngeneic marrow plus non-T-cell depleted allogeneic marrow has been found to produce complete allogeneic chimerism while avoiding GVHD. This model has been found to have potential usefulness in the treatment of leukemia, since the procedure does not appear to eliminate the graft-vs-leukemia (GVL) effect.

In order to decrease the toxicity of ablative radiation regimens used to prepare radiation chimeras, the use of monoclonal anti-T-cell subset antibodies as a substitute for part of the irradiation has been explored. Treatment with such antibodies in vivo has been found to deplete mature T-cells effectively from all peripheral lymphoid tissues, but not from the thymus. Selective thymic irradiation has therefore been added to this regimen, and has produced long-term mixed chimerism and specific tolerance across an MHC barrier with a non-lethal preparative regimen.

3. GENETICS OF THE MINIATURE SWINE MHC

Partially inbred strains of miniature swine, NIH mini-pigs, have been developed over the past fifteen years by this laboratory as a large animal pre-clinical model for transplantation studies. Five new recombinant haplotypes have been identified within these herds, and three of these have been bred to homozygosity. By serologic, biochemical and genetic data all of these recombinants appear to have split class I from class II loci.

Using the new recombinant MHC haplotypes, the relative importance of class I versus class II antigens for allograft survival has been examined. Both class I and class II antigens were found sufficient to produce prompt rejection of skin. However, class II antigen matching appeared to be of overwhelming importance in determining the outcome of vascular allografts such as kidney transplants (5023).

4. MOLECULAR BIOLOGY OF PORCINE CLASS II GENES

Because of the importance of class II antigens to renal allograft survival, a molecular approach to the analysis of class II genes in this species has been initiated (5023). DNA from each of the independent MHC haplotypes has been examined by extensive RFLP analysis using probes for human class II α and β genes. Hybridization with the human DQα cDNA probe revealed a single fr[illegible] in each of the enzymatic digests performed, and showed limited but definite [illegible] morphism. Hybridization with the human DRα cDNA probe showed a set of bands clearly distinct from those revealed with DQα, and showed no polymorphism among the herds. In contrast to the unique hybridization patterns of class II α probes, hybridization with class II β probes showed extensive cross hybridization. Polymorphism between all three haplotypes was apparent in many of the digests.

A genomic library has been constructed from the SLA^c lymphocyte DNA, and cDNA libraries have been prepared from SLA^c and SLA^d spleen message. These libraries have been screened with human class II cDNA probes, and clones analogous to many of the human class II α and β genes have been identified.

A surprising result was obtained when the sequence of the SLA $DR\beta^d$ cDNA gene was compared with a panel of human $DR\beta$ gene sequences. A comparison of the amino acid sequences showed that all three hypervariable regions of the $DR\beta^d$ allele were more similar to the corresponding regions of the human $DR1\beta$ allele than were the sequences of any other human $DR\beta$ allele. At the DNA level, the frequency of silent third base pair substitutions by which the pig $DR\beta^d$ and the human $DR1\beta$ differed was no different from that seen for comparison with other human $DR\beta$ alleles. These results suggest a high level of selection for this polymorphism at the protein level between these widely disparate species. Since class II genes are thought to be involved in the presentation of foreign antigens to the immune system, these results may imply that swine and human beings have both selected this allele because of its utility in the response to a common pathogen during evolution.

5. STUDIES OF BONE MARROW AND ORGAN TRANSPLANTATION IN MINIATURE SWINE

Without exogenous immunosuppression, miniature swine matched for class II and transplanted across a class I plus minor antigen difference have been found to frequently develop specific systemic tolerance. The mechanism of this tolerance has been investigated in vitro (5023) and has been found to involve a specific depletion of class I reactive helper T-cell populations. Also, consistent with a lack of T-cell help is the fact that recipients of class II matched renal allografts which went on to accept such grafts long-term showed a brief rejection crisis at approximately fourteen days during which there was a rise in IgM antibodies to SLA class I antigens, which subsided spontaneously without conversion from IgM to IgG.

Because the response to ablative regimens in miniature swine is much more similar to that of man than is the response of rodents, establishment of a bone marrow transplant regimen in this model has been carried out as a pre-clinical step (5023). Ablative radiation regimens have been established similar to those used for HLA identical sibling transplants in man, and have been found satisfactory to permit MHC matched bone marrow allografts. A new regimen with fractionated irradiation has been found necessary to permit successful transplantation of MHC mismatched bone marrow. This regimen has been found not sufficient to permit T-cell depleted bone marrow grafts to be accepted. Non-T-cell depleted bone marrow grafts across full MHC barriers were found to produce aggressive lethal GVHD. Addition of T-cell depleted autologous bone marrow to the non-T-cell depleted allogeneic inoculum has been attempted and, in some cases, has permitted engraftment while avoiding lethal GVHD. These data may have potential applicability to clinical transplantation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05021-17 I

PERIOD COVERED

October 1, 1987 to September 30, 1988

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:	T. M. Sundt III	Medical Staff Fellow	IB, NCI
	C. H. Chester	Guest Researcher	IB, NCI
	Y. Sharabi	Visiting Fellow	IB, NCI
	N. Shinohara	Visiting Scientist	IB, NCI
	M. Sykes	Visiting Associate	IB, NCI

COOPERATING UNITS *(if any)*

S. L. Epstein, Senior Staff Fellow, National Center for Drugs and Biologics, FDA

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	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.)*

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; 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; 5) Mixed allogeneic and xenogeneic chimeras: Irradiated animals are reconstituted with mixtures of T-cell depleted donor and host marrow, and the mechanism of tolerance and of immune responsiveness in these animals is studied in vivo and in vitro; 6) Non-lethal preparative regimens for bone marrow transplantation: Attempts to replace lethal irradiation with monoclonal antibody treatment in vivo are examined, and the immune status of the resulting animals is studied; and 7) Graft-vs-leukemia (GVL) effects of mixed bone marrow reconstitution: The effects of reconstitution with T-cell depleted syngeneic plus non-T-cell depleted allogeneic bone marrow on syngeneic leukemia are examined.

GPO 914-918

Project Description

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) Using a new MHC recombinant strain (C3H.KBR) we have detected an unusually high responsiveness to Qa antigens. Hybridomas prepared using appropriately immunized spleen cells from this strain yielded 11 new monoclonal antibodies directed against several epitopes of the Qa antigens. Immunoprecipitation studies using these antibodies indicate that at least two different molecules are detectable. In vivo injection of these antibodies has also been found to have profound effects on circulating T-cells. Two of these antibodies of the same immunoglobulin class but directed toward different epitopes of Qa-2 have been found to have different in vivo T-cell depletion characteristics. In vitro studies show that the antibody causing rapid depletion of T-cells in vivo causes much less cell surface modulation of Qa-2 than does the antibody leading to slow depletion. This difference may indicate that the antibody causing modulation reacts with the site on Qa-2 involved in its physiologic ligand binding.

3) A new recombinant which occurred during the backcrossing of A.TH has permitted, for the first time, an analysis of A.TH anti-A.TL reactivity in the absence of a Qa-1 antigen difference. Our results with skin grafting experiments indicate that in the absence of a Qa-1 difference, there is marked prolongation of skin graft survival. These studies suggest that a class II difference alone may be insufficient to cause skin graft rejection, and that class II antigens may actually function by presenting other antigenic differences (e.g., minor antigens or Qa antigens) to the immune system.

4) Anti-idiotypic antibodies reactive with monoclonal anti-H-2 and anti-Ia antibodies have been produced and shown to be specific by a variety of assays. These anti-idiotypic reagents have been used for in vivo manipulation of transplantation immunity. They have been found to induce idiotype without immunization by conventional antigen, and also to be capable of changing the humoral immune response so that an idiotype becomes predominant. However, no effect on T-cell immunity to MHC antigens has so far been found. For this reason monoclonal antibodies directed to T-cell receptors against MHC antigens are presently being developed.

5) 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. In vitro assays have been performed in order to examine the mechanism of transplantation tolerance in these animals. Evidence

for generation of high levels of natural suppressor (NS) cells in the early weeks following grafting has been obtained. Characterization of these suppressor cells by antibody reactivity indicates that they are not T cells. They are derived predominantly from the host component of mixed marrow grafts and may be involved in the elimination of those alloreactive cells which otherwise would cause GVH.

6) Because in vitro assays using spleen cells from mixed chimeras showed maximal suppressive activity at approximately 8 days following radiation and bone marrow reconstitution, delayed administration of non-T-cell-depleted allogeneic marrow to animals irradiated and reconstituted with T-cell-depleted syngeneic marrow was performed. This procedure was found to markedly reduce the GVH produced by allogeneic T cells and to result in long-term completely allogeneic chimeras.

7) 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. Experiments in which the syngeneic component of mixed marrow reconstitutions has been derived from sensitized animals and in which the hosts have also previously been sensitized indicate that the mixed marrow reconstitution regimen may be capable of overcoming transplantation across MHC barriers in a sensitized host.

8) Elimination of T cells from the syngeneic component, but not the allogeneic component of a mixed bone marrow inoculum has been found to produce completely allogeneic chimeras which are, however, protected from lethal GVHD. By adding the EL4 murine leukemia to the syngeneic component of such a reconstituting inoculum, it has been found that this procedure does not eliminate the strong graft-vs-leukemia (GVL) effect of non-T-cell depleted allogeneic bone marrow. The GVL effect has also been seen when non-T-cell depleted allogeneic marrow was added as late as 8 days following the T-cell depleted syngeneic marrow plus leukemia.

9) Using a mixture of anti-CD4 plus anti-CD8 monoclonal antibodies in vivo, transient bone marrow engraftment of allogeneic bone marrow was achieved with a total body irradiation dose as low as 300R. Addition of 700R thymic irradiation to this regimen led to long-term mixed chimerism and allogeneic skin graft tolerance.

Proposed Course of Project: Our backcrossing program for maintenance of congenic resistant strains will be continued, and intercross progeny will continue to be screened for useful intra-MHC recombinants. RFLP analysis of such recombinants using a battery of MHC-linked probes will be used to pinpoint and characterize new recombinants. Functional properties of new monoclonal anti-Qa antibodies will be examined in vitro and in vivo. Further attempts to characterize cells responsible for suppression of alloreactivity in mixed chimeras will include in vitro assays of specificity of suppression of CTL generation and in vivo attempts to adoptively transfer tolerance and GVH suppression. Attempts to extend studies of the GVL effect of mixed bone marrow reconstitution to additional syngeneic leukemias and established leukemias will be made. The use of monoclonal antibodies as a supplement or an alternative to lethal irradiation in host preparation for bone marrow transplantation will be examined.

Publications:

Sachs DH, Sykes M. Reconstitution of lethally irradiated mice with mixed bone marrow inocula: Prevention of GVHD and induction of tolerance. In: Speck B, Gratwohl A., eds. Bone Marrow Transplantation. Hampshire: The Macmillan Press Ltd, 1987;9-10.

Shinohara N, Hozumi N, Watanabee M, Bluestone JA, Johnson-Leva R, Sachs DH. Class II antigen specific murine cytolytic T lymphocytes (CTL). II. Genuine class II specificity of Lyt-2^+ CTL clones. J Immunol 1988;140:30-6.

Sykes M, Sachs DH. Mixed allogeneic chimerism as an approach to transplantation tolerance. Immunol Today 1988;9:23-7.

Sykes M, Chester CH, Sachs DH. Mixed bone marrow chimeras as an approach to transplantationn tolerance. In: David CS, ed. H-2 Antigens: Genes, Molecules, Function. New York: Plenum Press, in press.

Sykes M, Sachs DH. Mechanisms of suppression in mixed allogeneic chimeras. Transplantation, in press.

Sachs DH. Anti-idiotype to MHC receptors - A possible route to specific transplantation tolerance. In: Kohler H, Bona C., eds. International Reviews Immunology Vol 3. New York: Harwood Academic Publishers GmbH, 1988, in press.

Sykes M, Chester CH, Sachs DH. Protection from GVHD in fully allogeneic chimeras by prior administration of T-cell depleted syngeneic bone marrow. Transplantation, in press.

Sykes M, Eisenthal A, Sachs DH. Mechanism of protection from graft-vs-host disease in murine mixed allogeneic chimeras: I. Development of a null cell population suppressive of CML responses and derived from the syngeneic bone marrow component. J Immunol 1988;140:2903-11.

Hirsch R, Eckhaus M, Auchincloss H Jr, Sachs DH., Bluestone JA. Effects of in vivo administration of anti-T3 monoclonal antibody on T cell function in mice. I. Immunosuppression of transplantation responses. J Immunol 1988;140:3766-72.

Hirsch R, Eckhaus M, Auchincloss H Jr, Sachs DH, Bluestone JA. Effects of in vivo administration of anti-T3 monoclonal antibody on T cell function in mice. I. Immunosuppression of transplantation responses. J Immunol, in press.

Sykes M, Sharabi Y, Sachs DH. Achieving alloengraftment without graft-vs-host disease: Approaches using mixed allogeneic bone marrow transplantation. Bone Mar Transplant, in press.

Sykes M, Sheard MA, and Sachs DH. Effects of T-cell depletion in radiation bone marrow chimeras: 2) Requirement for allogeneic T-cells in the reconstituting bone marrow inoculum for subsequent resistance to breaking of tolerance. J Exp Med, in press.

Epstein SL, Sharrow SO, Sachs DH. Genetics of the induction of antigen-specific immunity in anti-idiotype treated mice, and derivation of monoclonal Ab3 antibodies. Annales d'Immunologie (Institut Pasteur), in press.

Chester CH, Sykes M, Sachs DH. Multiple mixed chimeras: Reconstitution of lethally irradiated mice with syngeneic plus allogeneic bone marrow from multiple strains. Transplantation, in press.

Sykes M, Sheard M, Sachs DH. Effects of T-cell depletion in radiation bone marrow chimeras: 1) Evidence for a donor cell population which promotes alloengraftment without the potential to produce GVHD. J Immunol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05023-17 I

PERIOD COVERED

October 1, 1987 to September 30, 1988

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:	E. O. Kortz	Medical Staff Fellow	IB, NCI
	K. Pratt	Senior Staff Fellow	IB, NCI
	T. M. Sundt III	Medical Staff Fellow	IB, NCI
	T. Suzuki	Visiting Fellow	IB, NCI
	F. Hirsch	Guest Researcher	IB, NCI
	P. C. Guzzetta	IPA Investigator	IB, NCI

COOPERATING UNITS *(if any)*

NIH Animal Center, Poolesville, Maryland
J. K. Lunney, Research Chemist, USDA Animal Parasitology Institute, Beltsville, MD
S. A. Rosenberg, Chief, Surgery Branch, NCI

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology 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 space provided.)*

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 human transplantation; 2) Assessment of the immunologic parameters involved in tolerance to allografts in this species; 3) Detection and characterization of intra-MHC recombinants: Five intra-MHC recombinants have been obtained and have been or are being 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; 4) 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; 5) 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; and 6) Analysis of class II MHC genes: Southern blot analyses using cDNA probes from human class II genes have been performed and indicate that genes corresponding to each of the major human class II loci are present in the pig genome. In addition, a genomic library and two cDNA libraries have been constructed and screened with these probes. Class II genes from the pig herds have been isolated and are being characterized and used in in vitro and in vivo transfection studies.

GPO 914-916

Project Description

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 five lines bearing intra-MHC recombinants.

2) Transplants of kidneys between recombinant and non-recombinant haplotypes have permitted an evaluation of the effects of selective class I and/or class II matching on vascular allograft survival. A non-MHC-linked gene determining immune responsiveness has been detected and determined to control rejection of vascular grafts across non-MHC differences. In animals bearing the non-rejector gene at this locus, vascular grafts are accepted across minor differences and also across class I plus minor differences, but not across class II differences. These studies indicate, therefore, that class II matching is of major importance for survival of vascular grafts.

3) Skin grafts are promptly rejected across class I or class II differences. However, following successful kidney transplants in the class II matched situation, donor skin grafts are specifically prolonged, indicating that acceptance of the vascular graft induces systemic tolerance.

4) The mechanism of systemic tolerance in class II matched kidney allografts has been examined. Studies on the generation of CTLs in vitro indicate that such tolerant animals cannot generate a CTL response across a class I only difference, as can ltheir non-grafted counterparts. The defect appears to involve helper independent class I reactive CTLs, since addition of other helper cells (such as anti-class II reactive populations) leads to normal anti-class I CTL activity. In addition, non-T suppressor cells have been identified as contributing to the defect.

5) Southern blot analyses of lymphocyte DNA from each of the miniature swine herds and recombinant lines have been performed. The data indicate that swine possess a single class II α chain gene corresponding to that of the human DR α chain, DQ α chain, and DP α chain. Of these, only the DQ α chain appears to be polymorphic within these herds. Approximately 5-7 class II β chain genes have been revealed by these analyses, which show both polymorphism and extensive cross-hybridization between β chains of the three major loci.

6) A complete EMBL-3 phage library has been constructed from the c haplotype, and class II genes corresponding to those detected in the Southern blot analysis have been isolated and characterized.

7) cDNA libraries from the c and d haplotypes have been constructed and screened with human class II probes. cDNA clones corresponding to $DQ\alpha^c$, $DQ\beta^c$, $DQ\alpha^d$, $DQ\beta^d$, $DR\alpha^d$, and $DR\beta^d$ have been isolated. Several of these genes have been successfully transfected into mouse L cells, leading to cell surface expression of the swine class II antigens on these cell lines.

8) Analysis of the derived amino acid sequence of the SLA $DR\beta^d$ cDNA gene has led to a surprising finding. Comparison of this sequence with sequences of a panel of human DRβ genes indicates that at the amino acid level all three hypervariable regions of the swine sequence are more similar to the corresponding regions of

the human DR1β allele than are the sequences of any other human DRβ alleles. At the DNA level, silent substitutions show no differences between different human β alleles. These data therefore suggest a high level of selection leading to the amino acid sequence homology between these species.

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 SLAdd haplotype, at least two others react with selective T-cell subpopulations, and at least one antibody is reactive with macrophages. Further characterization of these antibody reactivities is in progress.

10) A protocol which permits bone marrow engraftment across MHC differences has been obtained. Using this protocol, fully allogeneic bone marrow grafts led to lethal GVHD. Using a mixture of T-cell depleted autologous bone marrow plus non-T-cell depleted fully MHC mismatched bone marrow, alloengraftment has been obtained in 17 of 19 animals to date. In 9 of these animals clinical GVHD was self-limited, with resolution by the third week. These data indicate that, as in the murine model, T-cell depleted autologous bone marrow has an anti-GVHD effect when administered as part of the mixed bone marrow reconstitution inoculum. The longest survivor to date has lived 154 days, dying of probable radiation damage to the lungs.

Proposed Course of Work: In vitro assays will be performed to determine the nature of the observed suppression of CTL generation noted in recipients of class II matched kidney allografts. Exhaustive screening of genomic and cDNA libraries will be continued to ensure identification of all expressed class II genes in this model. Characterization of these class II genes with respect to sequence and expression will be performed, and construction of retroviral vectors to enable transfection of bone marrow cells with these genes will be initiated. Using new preparative regimens which permit allogeneic bone marrow engraftment, induction of tolerance by mixed bone marrow reconstitution in this large animal model will be attempted. Effects of mixed bone marrow reconstitution on transplant tolerance will be examined. Attempts will be made to decrease radiation damage to the lungs by substituting other modalities (cytoxan and/or monoclonal antibodies) for part of the total body irradiation. New hygienic measures will be attempted to improve the survival rate during the period of aplasia and transient GVHD.

Publications:

Sakamoto K, Pennington LR, Popitz-Bergez FA, Pescovitz MD, Gress RE, McDonough MA, Shimada S, Katz SI, Sachs DH. Swine GVHD model and the effect of T cell depletion of marrow by monoclonal antibodies. In: Gale RP, Champlin R, eds. Progress in Bone Marrow Transplantation Vol 53. New York, Alan R. Liss, Inc, 1987;449-53.

Moses RD, Clark RE, Lucas PJ, Quinones RR, Yeakel KS, Sachs DH, Gress RE. Immunological reconstitution and cardiac allograft survival in Rhesus monkeys conditioned with irradiation and T cell-depleted autologous marrow transplantation. In: Gale RP, Champlin R, eds. Progress in Bone marrow Transplantation Vol 53. New York, Alan R. Liss, Inc, Vol. 53, New York, Alan R. Liss, Inc, 1987;983-7.

Gustafsson K, Widmark E, Jonson A-K, Servenius B, Sachs DH, Larhammar D, Rask L, Peterson PA. Class II genes of the human major histocompatibility complex. Evolution of the DP region as deduced from nucleotide sequences of the four genes. J Biol Chem 1987;262:8778-86.

Bach FH, Sachs DH. Current concepts--immunology: Transplantation immunology. N Engl J Med 1987;317:489-492.

Pennington LR, Sakamoto K, Popitz-Bergez F, Pescovitz MD, McDonough MA, MacVittie, TJ, Gress RE, Sachs DH. Bone marrow transplantation in miniature swine. I. Development of the model. Transplantation 1988;45:21-26.

Popitz-Bergez FA, Sakamoto K, Pennington LR, Pescovitz MD, McDonough MA, MacVittie, TJ, Gress RE, Sachs DH. Bone marrow transplantation in miniature swine. II. Effect of selective genetic differences on marrow engraftment and recipient survival. Transplantation 1988;45:27-31.

Gress R, Moses R, Suzuki T, Lowman M, Pennington L, Sakamoto K, Sachs DH. Biparental bone marrow transplantation as a means of tolerance induction. Transplant Proc 1987;19:95-97.

Sakamoto K, Sachs DH, Shimada S, Popitz-Bergez FA, Pennington LR, Pescovitz MD, McDonough MA, MacVittie TJ , Katz SI, Gress RE. Bone marrow transplantation in miniature swine. III. Graft-versus-host disease and the effect of T cell depletion of marrow. Transplantation 1988;45:869-875.

Sachs DH, Germana S, El-Gamil M, Gustafsson K, Hirsch F, Pratt K. Class II genes of miniature swine. I. Class II gene characterization by RFLP and by isolation from a genomic library. Immunogenetics 1988;28:22-29.

Sachs DH, Suzuki T, Sundt T, Sykes M. A new approach to bone marrow transplantation across MHC barriers. In: Gale RP, Champlin R eds. Bone Marrow Transplantation: Current Controversies, Vol 91. New York: Alan R. Liss, Inc., 1988, in press.

Pescovitz MD, McDonough MA, Sachs DH. Expression of T cell associated antigens by porcine natural killer cells. Immunology, in press.

Kortz EO, Sachs DH. Suppression of CML reactivity by non-T-cells from miniature swine tolerant of a class I mismatched renal allograft. Current Surgery, in press.

Sundt TM III, Suzuki T, Kortz EO, Eckhaus M, Gress RE, Sachs DH. Induction of specific transplant tolerance in a large animal model by bone marrow transplantation. Surgical Forum Volume, in press.

SUMMARY REPORT
EXPERIMENTAL IMMUNOLOGY BRANCH
October 1987 - September 1988

The Experimental Immunology Branch carries out laboratory investigations in basic immunobiology with particular emphasis in the following areas: 1) lymphocyte differentiation and regulation; 2) cell biology of immune responses; 3) transplantation biology; 4) structure, regulation and function of genes involved in the immune response; 5) tumor immunology; and 6) flow cytometry. This report briefly summarizes research efforts in each of the foregoing areas during the past year. More detailed information on specific accomplishments can be found in the individual annual reports cited by number in the text.

1. LYMPHOCYTE DIFFERENTIATION AND REGULATION

Dr. Gene Shearer's laboratory has investigated a series of murine models of autoimmunity with lupus-like characteristics, including a graft versus host (GVH)-induced model of lupus, the genetically determined lupus-like syndrome in MRL/lpr mice, and a retroviral induced model of B cell lymphoproliferative disease (9282). All three models are characterized by autoantibody production and activation of suppressor T cells that are selective in their effect on $CD4^+$ T helper cells. Preliminary studies in humans with SLE indicate that most of these patients exhibit a selective defect in $CD4^+$ T helper function. These studies may provide a better understanding of immune regulation and dysregulation in autoimmune disease.

Dr. Shearer's laboratory has also investigated the effects of different immunologic insults on the development of murine T lymphocytes from bone marrow stem cells (9264). These studies indicate that either a GVH reaction or treatment of mice with cyclosporin A (CsA) results in thymic damage such that after irradiation and bone marrow grafting, both $CD4^+$ and $CD8^+$ cells develop, but there is a selective absence of $CD4^+$ T helper function. If bone marrow transplantation precedes treatment with CsA, mice fail to develop single positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) T cells. These results provide a model that should be useful for investigating: a) the role of the thymus in T cell development; b) the expression of T cell receptors; and c) the effects of GVH and CsA on immune reconstitution in patients exposed to these immunologic insults.

Studies of T lymphocyte responses in HIV^+, asymptomatic individuals by Dr. Shearer's laboratory indicate that approximately 50% of these patients exhibit selective $CD4^+$ T helper cell functional defects without any AIDS symptoms and without loss of $CD4^+$ cell numbers (9267). A defect of antigen-presenting cell (APC) function was not observed in any such asymptomatic individuals. Many patients in various stages of disease exhibited a selective loss of $CD4^+$ T helper function but retained $CD4^-$ T helper function to HLA alloantigens. The results of this study: a) permit the detection of three stages of T helper dysfunction not previously defined by AIDS diagnostic tests; b) do not implicate an APC defect early in disease progression; and c) provide evidence for

an alternate ($CD4^-$) T helper pathway that may be utilized for developing immunotherapy strategies in HIV^+ patients.

The role of the T cell receptor (TCR) $\alpha\beta$ heterodimer in antigen recognition has been analyzed in studies carried out in the laboratory of Dr. Richard Hodes (9265). T cell clones specific either for alloantigen or for conventional peptide antigens in association with self major histocompatibility complex (MHC) determinants were analyzed. When the crossreactive repertoires of these clones were studied, a strong and selective association was observed such that most clones specific for the peptide 81-104 of cytochrome c were crossreactive to the Mls^c alloantigen. An evaluation of TCR usage in panels of clones as well as heterogeneous T cells revealed that the basis for this specificity lies in the fact that expression of the $V\beta3$ gene segment is sufficient to confer Mls^c specificity on a T cell. In addition, it was demonstrated that mouse strains which express Mls^c delete expression of $V\beta3$ in their mature peripheral T cell populations. This failure to express $V\beta3$ presumably reflects a consequence of the maintenance of tolerance to self Mls^c products. These findings provide a basis for the understanding of high T cell precursor frequency for Mls^c products, reflecting the overall expression of $V\beta3$ in the T cell repertoire. Moreover, they demonstrate the importance of these products in selection of the antigen-specific T cell repertoire through a mechanism which appears to involve the clonal deletion of potentially self-reactive T cells.

The role of endogenous lymphokines in T cell activation has also been evaluated in Dr. Hodes' laboratory (9281). The proliferative responses of type 2 T helper clones are inhibited by antibody specific for the lymphokine interleukin 4 (IL4), which is produced by these clones. This demonstrates that IL4 acts as an autocrine growth factor for these T cells. In addition, mRNA levels corresponding to a number of activation genes are inhibited by the presence of anti-IL4 antibody during T cell activation. In contrast, the level of IL4 mRNA actually increases in the presence of this antibody, suggesting that endogenously produced IL4 may normally down regulate its own steady state mRNA levels.

A new monoclonal antibody produced in Dr. Dickler's laboratory recognizes a previously undescribed mouse alloantigen (Ly-39.1) which is preferentially expressed on B lymphoblasts. The expression of this molecule is controlled by a gene located centromeric to H-2 on chromosome 17. This antibody may allow efficient separation of resting and activated B lymphocytes (9277).

An assay developed in Dr. Dickler's laboratory utilizes particle concentration fluorescence and has the capability of screening for monoclonal antibodies which detect cell surface molecules at very low levels (100-300 molecules per cell). This assay should be useful in detecting previously unrecognized cell surface molecules (9277).

Dr. Segal's laboratory has established that all of the cytotoxic activity in normal peripheral blood T cells induced by anti-CD3 containing heteroconjugates resides in the CD8+,CD4- subset. They have found that this activity can further be localized to the light fraction on a Percoll density gradient, consisting of CD3+, CD8+, Leu-7+ cells. These cells are extremely potent

cytolytic effectors since they comprise less than 5% of the lymphocytes. The vast majority of T cells residing in the Percoll dense fraction have no cytolytic activity on day 0 or after 1-2 days of stimulation with IL-2. However, 5 days of stimulation with IL-2 plus anti-CD3 bound to the floor of the tissue culture flask induces both proliferation and the generation of targetable cytolytic effector cells (9254).

Dr. Shaw's laboratory has continued to characterize two major subsets of human T lymphocytes which they have determined to be naive and memory T cells (9257). These subsets differ in major ways both in phenotype and function. Phenotypically, there are differences between subsets in expression of at least 6 different cell surface molecules including three involved in adhesion (CD2, LFA-3, and LFA-1). Functionally, only the memory cells respond to recall antigen. In addition, the memory cells are much more responsive to CD3 mAb-mediated triggering than are naive cells, and only memory cells respond to CD2 mAb mediated stimulation. Enhanced responsiveness to receptor-mediated triggering is a novel mechanism for T cells which could facilitate memory cell response to specific antigen. Furthermore, the memory cells produce much more gamma interferon in response to a variety of stimulatory agents than do naive cells.

Studies by Susan Sharrow have demonstrated that cell surface expression of the MHC Class I antigen Qa-2 by murine thymocytes uniquely identifies, within the thymus, subsets of T cell receptor-bearing cells with differentiated phenotypes. Because all peripheral T cells are Qa-2^+, this result suggests that Qa-2 positive thymocytes represent those T cells ready for export from the thymus and that the final stages of thymocyte differentiation are more complex than those postulated in current models of T cell development (9255).

Studies from Dr. Al Singer's laboratory have demonstrated that confrontation with thymic Ia determinants is necessary for the development of class II-specific $CD4^+$ T cells, but is not necessary for class II-specific $CD8^+$ T cells (9268). This observation indicates that the differentiation requirements of phenotypically distinct T cell subsets are not identical. Dr. Al Singer's laboratory has continued to characterize the differentiation and recognition specificities of $CD8^+$ class I specific T helper cells (9256). It was found that these T cells differentiate intrathymically and are tolerized by thymic MHC determinants, but display a remarkably narrow response repertoire that so far includes only class I MHC alloantigens and viruses.

The roles performed by CD4 and CD8 molecules in T cell function have also been examined in Dr. Al Singer's laboratory. It was found that CD8 performs a signalling function in the activation of $CD8^+$ T cells that requires multivalent cross-linking of CD8 (9268). However, challenging the concept that CD4 and CD8 are functionally equivalent molecules that differ in their MHC ligand specificity are studies demonstrating that CD4 and CD8 differ in their response to activation of protein kinase C.

The role of T cell receptor in T cell differentiation has been addressed in Dr. Al Singer's laboratory utilizing genetically mutant mice with severe combined immunodeficiency (scid) (9273). SCID mice fail to express T cell receptors, and it was found that this results in a failure of scid thymocytes to

express CD4 and/or CD8, indicating a relationship between TcR expression and CD4/CD8 expression. To better analyze events in T cell differentiation, Dr. Al Singer's laboratory has also examined thymocytes that have differentiated in vitro in thymic organ cultures for function. It has recently been found that such in vitro generated thymocytes are able to function both as lymphokine secreting T helper cells as well as cytolytic effector cells.

2. CELL BIOLOGY OF IMMUNE RESPONSES

Dr. Hodes' laboratory has analyzed the mechanisms mediating the function of T helper cells in T dependent B cell antibody responses (9266). A role of both specific and non-specific T cell derived signals was demonstrated for activation of B cell antibody responses. It was shown that the supernatants of activated cloned T helper cells were capable of replacing these T cells in the antigen specific and MHC restricted induction of B cell IgG antibody responses. Two distinct and synergizing activities were identified in this supernatant: Interleukin 4, and an antigen-specific and MHC-restricted soluble factor. It was demonstrated that this latter factor can be affinity purified employing monoclonal antibodies specific for T cell receptor Vβ8 determinants. A mechanism for the rapid and directed secretion of mediators by T help was also demonstrated by analysis of granule exocytosis by T helper cells. Cloned helper cells, in response to T cell receptor mediated stimuli but not in response to IL2, rapidly secrete the contents of cytoplasmic granules. This provides a potential mechanism for the transmission of B cell activating stimuli from helper T cells to target B cells.

Dr. Segal's laboratory has shown that antigen-presenting cells coated with several different antibody heteroconjugates present antigen to T cells at concentrations which would normally be too low to elicit a response (9254). The presentation of antigen to T cells can lead to the expansion and activation of antigen-specific T-helper clones, which in turn are essential for the generation of cellular and humoral responses against that antigen. By using the TA3 B cell hybridoma as APC, they found that antigen targeted to both class I and class II MHC antigens was presented 500-1000 times more efficiently than untargeted antigen, and that targeting to surface immunoglobulin and $Fc_\gamma R$ also improved the efficiency of presentation, but to a lesser degree. With splenic B cells, heteroconjugates containing anti-sIg, anti-class I and anti-class II, but not anti-$Fc_\gamma R$ gave enhanced presentation, whereas effective antigen targeting to the adherent cells was seen only when antigen was targeted to class I or class II molecules. Presentation of targeted antigen by B cells requires a time dependent processing step. Antigen targeted to surface immunoglobulin was endocytosed and presented more rapidly than antigen targeted to MHC molecules. Preliminary studies in Dr. Segal's laboratory have shown that heteroconjugates can aid in the development of antibody responses in vivo. Heteroconjugates containing anti-class I or anti-class II, but not anti-IgD antibodies were able to enhance antibody production, suggesting that the enhancement was not occurring via B cells. Controls containing a mixture of the non-conjugated antibodies, or either antibody of the heteroconjugate alone did not enhance priming.

Studies on the mechanism of lymphocyte-mediated cytotoxicity in Dr. Henkart's laboratory (9251) have shown that red blood cells are killed by both cloned

cytotoxic T lymphocytes and helper T cells when linked to these cells by their T cell receptor. This cytotoxicity appears to operate by the same mechanism as the killing of more complex nucleated targets. Studies on the potent lytic agent cytolysin isolated from the secretory granules of cytotoxic lymphocytes have revealed that this protein can bind to target cells in a step prior to the lytic event. The lysis of the stable intermediate produced after this binding requires calcium. Studies on tumor target cells show that cytolysin can cause a sublethal membrane damage which can be repaired by the cells.

Studies in Dr. Shaw's laboratory continue to be directed at elucidating the mechanisms of T cell recognition, with particular emphasis on antigen-independent T cell adhesion as a critical early step in the process of recognition (9257). Major progress has been in understanding the functional role of the ICAM-1 molecule which their previous functional studies have suggested functions as a ligand for LFA-1. ICAM-1 has been purified by immunoaffinity chromatography. Each of two sites on ICAM-1 defined by monoclonal antibodies are implicated in its adhesion function. Furthermore, ICAM-1 must be an adhesion ligand per se since ICAM-1 immobilized on plastic mediates LFA-1 dependent adhesion of T, B and myeloid cells. This simplified system of cell binding to ICAM-1 allows isolation/analysis in a well defined system of important features of more complex cell interactions including characteristic divalent cation requirements, and a process of dissociation.

Dr. Henkart's laboratory (9263) has developed a model system to explore the possibility that lymphocyte granule exocytosis occurs into a synapse-like zone created between the lymphocyte and an attached triggering cell. When allowed to settle onto glass surfaces coated with anti-T3 antibody, cloned mouse CTL and T helper cells exclude the penetration of antibodies from a region of substrate underneath themselves. Most other combinations of antibodies and lymphocytes do not form such a zone of exclusion beneath the attached cell. The formation of these synapse-like junctions is not blocked by drugs which block energy production or interfere with cytoskeletal protein function, and thus may be formed by diffusion of membrane antigen.

The role of secretory granule proteases in mediating target cell DNA breakdown during killing by cytotoxic T lymphocytes has been examined by Dr. Henkart's laboratory (9263). By pretreatment of intact CTL with the covalent inhibitor PMSF granule proteases could be efficiently inactivated in the presence of agents which raise the pH of acidic intracellular compartments. This did not significantly inhibit the lytic efficiency of the CTL, but did greatly reduce target DNA breakdown. They have also found that cytotoxicity mediated by purified granules from CTL or NK tumors is accompanied by target DNA breakdown, and that this appears to require both cytolysin and another granule component. This component(s) has been studied by assaying DNA release from detergent-isolated nuclei. In CTL granules this activity is dramatically inhibited by low concentrations of PMSF and DFP, which are specific inhibitors of serine proteases. These suggest a role for serine proteases in target cell DNA breakdown.

Recent studies in Dr. Dickler's laboratory have shown that IL-4 specifically induces a marked (90%) loss of binding of complexes to B lymphocyte FcγR II (9253). The IL-4 induced loss of binding of complexes was time and tempera-

ture dependent and was reversible. The loss of binding of complexes did not appear to be due simply to a loss of expression of FcγR II because IL-4 induced only a moderate reduction in the binding of two FcγR II specific monoclonal antibodies. These results suggest that IL-4 regulates the binding of complexes to B lymphocyte FcγR II by inducing some form of alteration of the receptor. This effect could play a role in the regulation of B lymphocyte responses by preventing FcγR II mediated downregulation.

3. TRANSPLANTATION BIOLOGY

Studies from Dr. Shearer's laboratory (9264) have focused on the effects on T lymphocyte function of treatment of mice with cyclosporin A (CsA). Exposure of mice in vivo with different doses of CsA indicate that lower doses of CsA selectively abrogate $CD4^+$ T helper function and activate T suppressor cells that selectively inactivate $CD4^+$ T helper function, whereas higher doses of CsA abolish both $CD4^+$ and CD^+ T cell functions and activate non-T suppressor cells that inactivate all T cell functions tested. Preliminary studies in humans treated with different doses of CsA suggest similar patterns of T cell inactivation. These findings may be important for understanding the mechanism(s) responsible for the effects of CsA on allograft retention and may be useful in determining optimal doses of CsA for treatment of transplant patients.

Other experiments from Dr. Shearer's laboratory (9260) indicate that murine cytomegalovirus (MCMV) infection can synergize with a graft-versus-host (GVH) reaction to make the GVH more severe. MCMV was found to provide a class II-like signal to synergize with a class I GVH, but does not appear to provide a class I-like signal and synergize with a class II GvH. The antiviral drug DHPG can reduce MCMV infection, but does not reduce the severity of GVH in MCMV-GVH synergy. Studies of this type may help to elucidate the synergistic effects of CMV infection and GVH disease in human bone marrow transplantation.

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL), may play a significant role in mediating allogeneic marrow graft rejection. In a murine model system developed in Dr. Gress' laboratory (9287), it was found that: 1) a cloned CTL population was sufficient to reject an allogeneic marrow graft; and 2) the mechanism by which these marrow grafts were rejected was specific for MHC gene products expressed by the donor marrow corresponding to the cytotoxic specificity of the clone. One approach to inhibit such graft rejection by CTL is by in vivo administration of monoclonal antibodies with specificity for CTL surface antigens. Administration of anti-CD3 to mice results in T cell activation within hours of administration, manifest by secretion of colony stimulating factors (CSF) detectable in the serum and produces extramedullary hematopoiesis in the spleen. In considering the use of anti-CD3 monoclonal antibody to enhance the engraftment of allogeneic marrow, these results indicate that it may be beneficial to time the injection of allogeneic marrow so that it is present during the period of T cell activation induced by the antibody in order to provide the possible benefit of factors which stimulate hematopoiesis.

Studies in murine marrow chimeras have suggested that MHC mismatched marrow transplantation is associated with immunoincompetence of the T cell stem of the

immune response. To evaluate whether T cell maturation processes are comparable in mouse and man, the development of T cell responses in a primate, the rhesus monkey, has been studied in other experiments from Dr. Gress' laboratory (9288). The time course of $CD4^+$ T cell reconstitution and the time course of the development of in vivo T cell immunocompetence (as defined by the ability to respond to an organ allograft) correlated with the number of T cells infused in autologous marrow, and did not correlate with marrow cell dose or with time of hematopoietic reconstitution, raising the possibility that residual T cells in the infused T cell depleted marrow played a central role in the generation of subsequent T cell populations. This would imply that the generation of T cells in marrow grafted primates may not be the same as in the mouse. Such differences would potentially have important implications for the transplantation of HLA-mismatched marrow in man.

Recent studies in Dr. Al Singer's laboratory have examined the specificity of the cells effecting in vivo graft rejection responses (9275). Using allophenic mice as the donors of skin allografts, it was found that the effector mechanism of skin allograft rejection is mediated by T cells that assess individual cells in the dermis of the graft for expression of foreign histocompatibility antigens.

4. STRUCTURE, REGULATION AND FUNCTION OF GENES INVOLVED IN THE IMMUNE RESPONSE

Studies carried out in Dr. Dinah Singer's laboratory have demonstrated that the swine class I MHC multigene family contains only seven members, and as such is the smallest known class I family. Recent studies have been aimed at the characterization of the structure, expression, and function of this family. Physical mapping has established that all of the genes are linked and resident within 500 kb. Determination of the DNA sequences of five members of the family have shown that the sequence organization of the class I SLA genes is similar to that of other class I genes in other species. Despite the small size of the family, three sub-groups of genes can be discerned based on their sequence homologies (9269). Analysis of class I cDNA clones has established the exon organization of the genes, as well as their expression. Only four class I genes appear to be expressed in vivo (9269, 9270). The three subgroups defined by DNA sequence also display discrete patterns of expression. Thus, the genes of one sub-group are expressed ubiquitously, although their quantitative levels vary. A second sub-group defines a differentiation antigen. A third sub-group does not appear to be expressed at all (9270, 9276).

In order to investigate the molecular basis for the differential patterns of expression, Dr. Dinah Singer's laboratory has begun to generate and analyze transgenic mice containing isolated swine class I genes (9276). For one gene, encoding a classical transplantation antigen, it has been demonstrated that the pattern of expression of the swine gene in the transgenic parallels that observed in situ. Transgenics containing three other class I gene constructs have been generated.

Dr. Dinah Singer's laboratory has initiated studies to characterize DNA sequence elements and cognate trans acting factors associated with class I MHC genes (9270, 9276). A series of negative and positive regulatory DNA sequence

elements within the 5′ flanking region of the classical transplantation antigen gene have been identified. In particular a novel negative regulatory element has been described whose function is absolutely dependent on the presence of an enhancer element. This interaction is mediated by trans acting factors. Physical mapping of binding sites of trans acting factors has been achieved by both gel retardation and exonuclease protection assays. Although the molecular mechanisms by which the regulatory elements and their factor function is not known, the data suggest a model in which the negative and positive regulatory elements each define an independent binding site (9270).

Class I MHC gene expression is known to be induced by immunomodulators. Recent studies in Dr. Dinah Singer's laboratory have not only demonstrated the ability of a foreign class I gene in a transgenic mouse to respond to interferon, but have mapped the interferon responsive element. Furthermore, other studies in the same laboratory have demonstrated that ethanol, at physiologically attainable levels, is able to act as an immunomodulator by elevating steady state RNA and cell surface levels. Measurement of class I MHC antigen on peripheral blood lymphocytes in acutely intoxicated individuals showed a highly significant increase over controls (9270, 9276, 9285)

Dr. Kelly's laboratory has isolated over 60 novel cDNA clones which represent the immediate response of resting human peripheral blood T cells when activated by mitogen to proliferate (9274). This primary response is highly complex, both in terms of the number of inducible genes as well as the diversity of their regulation. Although a majority of the genes involved are shared with the activation response in normal human fibroblasts, a significant number are more restricted in their tissue specificity and thus may be encoding or effecting the differentiated functions of activated T cells. Surprisingly many inducible genes are inhibited by the immunosuppressive drug cyclosporin A, which is known to repress the transcription of several lymphokine genes. In addition, a subset of inducible genes are stimulated to constitutive expression in T cell clones infected with the immortalizing human retrovirus, HTLV I. Such abnormally-expressed genes may play a role in the deregulated growth of such cells.

Work in Dr. Kelly's laboratory to investigate the genetic regulatory mechanisms that govern tissue specific expression of the T cell receptor (TCR) β chain have shown that the nature of the vector containing a genomic β chain gene as well as the relative amount of 5′ untranslated sequence contained within a genomic clone influence the degree of tissue specificity observed in transfection assays (9286). TCR β chain genes containing 5 kb of 5′ UT sequence within a vector which contains polyoma enhancer and replication origin sequences display no tissue specificity of expression. By contrast, the same β chain gene within a pBR vector containing no eucaryotic regulatory sequences shows preferential expression as expected in T cells as compared to fibroblasts. Thus, unlike immunoglobulin genes, tissue specificity of expression as determined by resident regulatory sequences within a genomic TCR β chain gene are dominantly influenced by external eucaryotic regulatory sequences. In addition, TCR β chain genes containing 1.5 kb of 5′ UT sequence within a pBR vector display no preferential tissue expression. These results imply that a tissue-specific negative regulatory element exists within the deleted 3.5 kb of upstream sequence.

5. TUMOR IMMUNOLOGY

Drs. Segal and Wunderlich have shown that human T and K lymphocytes, targeted against tumors by heterocrosslinked antibodies, are able to prevent the establishment and growth of human tumors at low effector:target ratios (e.g. at 1:1 or less) in classic Winn-type tumor neutralization assays in nude mice (9254). Whereas all of the targeted T cell cytotoxicity as measured in a ^{51}Cr-release assay resided in the CD8+, CD4- subset, most of the tumor neutralizing activity was in the CD4+ subset. In order to study this discrepancy, they have established an in vitro tumoristasis assay. Results from these studies show that targeted PBL exhibit strong tumoristatic activity, and that much of this activity resides in the CD4+ subset. Preliminary evidence suggests that targeted tumoristasis may be mediated through a soluble factor.

Drs. Segal and Wunderlich have used human peripheral blood lymphocytes, targeted with heterocrosslinked antibodies and injected ip, to treat early intraperitoneal growth of a human ovarian adenocarcinoma cell line in nude mice (9250, 9254). They have found that targeted PBL from most donors can abrogate growth of established tumor cells. Since the human ovarian carcinoma cell line grows ip in nude mice in much the same way that the primary cancer grows in patients, these studies could form the basis for initiating clinical trials, using targeted PBL to treat ovarian cancer.

Recent studies in Dr. Wunderlich's laboratory have shown that trace amounts of chemicals related to lipopolysaccharides, although inactive alone, can serve as potent cofactors for in vitro induction of activated killer cells against tumors by macromolecular polyanions, such as dextran sulfate, fucoiden, and polyinosinic acid (9250). These findings offer alternatives to high doses of recombinant IL-2 for induction of activated killer cells.

Dr. Henkart's laboratory has developed a new general method for measurement of cytotoxicity, which should replace the standard ^{51}Cr release method for many purposes (9251). The method utilizes the fluorescent dye BCECF, which is loaded into target cells as an ester. The dye, released by target cells after incubations of 4 hours or less with cytotoxic effectors, is measured by a newly developed scanning microfluorimeter which reads a 96 well microtiter plate in about one minute. This technique is thus faster and cheaper than the ^{51}Cr method, and does not involve radioactivity.

Dr. Ting has shown that anti-CD3 augments the cytotoxic activity of murine cells activated by recombinant IL-2 and induces the generation of activated killer (CD3-AK) cells by triggering endogenous production of lymphokines (9272). CD3-AK cells react with a wide variety of tumor cells, as do conventional LAK cells. However, cytotoxic CD3-AK cells grow longer in culture than LAK cells and thus may be an ideal candidate for use in adjuvant adoptive immunotherapy of cancer.

6. FLOW CYTOMETRY

The Experimental Immunology Branch Flow Cytometry Laboratory is managed by Susan Sharrow (9255). The laboratory currently supports 60 individual

research projects for 45 investigators. These investigations involve multi-parameter quantitative single cell analyses of a variety of cell types freshly prepared from different species/tissues as well as a spectrum of in vitro cultured cells. Basic research support is provided to members of the EIB and the Immunology Branch as well as on a selected basis to other members of DCBD and investigators elsewhere at NIH who do not have access to appropriate flow cytometry instrumentation. Currently supported projects include a broad range of applications in the areas of cellular immunology, immunochemistry, transplantation biology, clinical immunology, immunogenetics, tumor immunology, and molecular biology. These include, but are not limited to, the following studies; a) in vivo and in vitro analyses of intra-cellular signalling via T cell surface molecules, b) characterization of T cells from animals with genetic or induced immune dysfunction; c) studies of the pathogenesis of graft-versus-host disease; d) analyses of the correlations between T cell memory and the quantitative expression of cell surface adhesion molecules, e) investigations of T cell ontogeny and differentiation, f) analyses of the mechanisms involved in marrow graft rejection versus acceptance, and f) analyses of regulation of expression of Class I genes.

During the past year, the EIB flow cytometry laboratory has implemented the instrument modifications and procedures necessary for performing three-color immunofluorescence analyses. In addition, a project to construct a Micro-VAX-based list mode data analysis work station has been implemented in collaboration with the Division of Computer Research and Technology.

Studies in the laboratory of Dr. Shaw have continued to define phenotypic characteristics of human naive and memory T cells (9257). The tissue distribution of a variety of new monoclonal antibodies has also been investigated by Dr. Shaw's laboratory and have resulted in presumptive identification of some antibodies as being against known molecules and suggestion that two are against previously undefined molecules (9257).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 09250-23 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 05003-22 I

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, laboratory, and institute affiliation)*

PI:	John Wunderlich	Senior Investigator	EIB, NCI
Others:	D. Segal	Senior Investigator	EIB, NCI
	C.C. Ting	Senior Investigator	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.9	1.9	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.)*

We have extended our investigation of tumor abrogation in vivo by human lymphocytes retargeted by heterocrosslinked antibodies to react with a human ovarian carcinoma cell line. Using an athymic mouse model in which intraperitoneal growth of established tumor is treated with retargeted human lymphocytes, cumulative results of a dozen experiments have confirmed that crosslinking the T-cell receptor complex, expressed by T lymphocytes, to tumor cells markedly enhances tumor abrogation relative to lymphocytes alone or to crosslinked antibodies alone. Moreover, results of a variety of additonal controls carried out in vivo have confirmed that the heterocrosslinked antibodies indeed facilitate tumor abrogation (collaborative effort with Dr. D. Segal, see project no. Z01 CB 09254-14 E).

Two aspects of our understanding of mouse activated killer (AK) lymphocytes, activated in vitro to react against a wide variety of tumors in a fashion not restricted by MHC, have been advanced this year. First, the mechanism(s) for generating AK cells against tumor cells has been clarified by finding that LPS-related molecules serve as potent cofactors for induction of AK cells by selected macromolecular polyanions, such as dextran sulfate, fucoiden, and polyinosinic acid. The cofactors are not independently active, insofar as they induce little or no AK cell activity alone and enzymatic digestion of the polyanions abrogates generation of AK cells. Second, we have markedly enhanced the veto activity that accompanies thymocytes with IL-2 induced AK cell activity, by optimizing both the activation conditions and their use with in vitro allogeneic responses subject to veto activity. Veto activity establishes tolerance to foreign tissues. We anticipate that these findings will facilitate activation of lymphocyte antitumor activity in vivo and establishment of tolerance to foreign cells in vivo.

Project Description

Major Findings;

The focus of this laboratory has been on antitumor cytotoxic effector cells and factors which effect their generation. This year we have extended our studies of a) retargeting of human lymphocytes to human tumor cells, and b) MHC-nonrestrictive cytotoxicity mediated by activated killer (AK) cells.

1. Retargeting the immune system against human tumor cells. In collaboration with Dr. David Segal we are continuing our efforts to focus the immune system against selective tumors by redirecting the specificity of lymphocytes with heterocrosslinked antibodies. Heterocrosslinked antibodies are generated by chemically crosslinking monoclonal antibodies which react on one hand with triggering sites on the lymphocytes and on the other hand with antigens selectively expressed by tumor cells. We are concentrating on treating an ovarian cancer cell line growing as an established tumor interperitoneally in immunocompromised athymic mice. Tumor growth mimics important features of the human disease by generating massive ascites and invasive tumor implants, which progress to pulmonary metastases and death by respiratory compromise and bowel obstruction.

Ovarian cancer cells are injected intraperitoneally into nude mice on day 0. Over a period of 48 hours between days 4-6, mice are treated with intraperitoneal injections of rIL-2-activated human lymphocytes from a normal donor with or without heterocrosslinked antibodies. Tumor cells are established and growing by 4 days after intraperitoneal injection as shown by a 70% increase in tumor cell number in the peritoneal lavage fluid. We estimate that the ratio of lymphocytes to tumor at the time of treatment is 1:1 or less. Tumor growth is monitored primarily by the amount of tumor in the peritoneal lavage fluid, collected on day 15. The lavage fluid from tumor-positive mice forms a large pellet following centrifugation, whereas the fluid from negative mice fails to form a pellet; this observation has been confirmed by ELISA assays for tumor-antigens in the lavage fluid.

Cumulative results of a dozen tests have demonstrated that heterocrosslinked antibodies which bind to triggering sites (CD3) on human T lymphocytes and tumor antigens on the ovarian cancer cells dramatically improve the antitumor effects of lymphocytes alone. The frequency of tumor-free mice, among 75-80 mice in each group, has been increased from 24 to 61% by treating tumor-bearing mice with heterocrosslinked antibodies and lymphocytes rather than lymphocytes alone. Lymphocytes from most but not all donors have protected against tumor growth when combined with heterocrosslinked antibodies; in three out of twelve experiments we have not observed protection.

A variety of controls suggest that heterocrosslinked antibodies work by crosslinking triggering sites on lymphocytes to tumor cells: 1) heterocrosslinked antibodies that protect against tumor growth in the presence of lymphocytes have failed to protect in the absence of lymphocytes; 2) heterocrosslinked antibodies that bind to tumor cells and to inactive sites (e.g. MHC)

rather than triggering sites on lymphocytes, have failed to protect against tumor growth; 3) heteroconjugates which bind to lymphocyte triggering sites but not to tumor cells have failed to protect; and 4) heterocrosslinked antibodies generated with Fab antibody preparations have protected as well as those generated with intact antibodies, showing that antibody binding to the Fc receptors on lymphocytes is not necessary for protection.

Tumor-selective monoclonal antibodies (113F1 and OVB3) have been identified that protect against growth of the ovarian carcinoma cell line when used in heterocrosslinked antibody preparations with lymphocytes. This finding is important because cocktails of heterocrosslinked antibodies against different tumor antigens should be useful in treating tumor populations with antigenic variants.

2. MHC-nonrestricted cytotoxicity of activated killer (AK) cells against tumors. Previous work in this laboratory has shown that potent AK cell activity against tumors is generated by culturing mouse lymphoid cells with selected macromolecular polyanions, such as polyinosinic acid. The response is dependent on two types of ancillary cells, accessory cells and helper cells, in addition to cytotoxic precursor cells. The response to polyanions does not simply involve enhanced generation of IL-2, a well-known inducer of activated killer cells, because a) we do not detect enhanced release of endogenous IL-2 by sensitive biological assays for cell-free IL-2, and b) there is a strain-dependent discordance in AK cell responses to recombinant IL-2 versus polyinosinic acid combined with a recombinant IL-2. Moreover, activation of cytotoxic cells is not mediated simply by interferon, insofar as we have not been able to duplicate the effects of polyanions by combinations of interferons α, β or γ.

This year we have found that trace amounts of lipid A or chromatographically purified phenol extracts of selected lypopolysaccharides (LPS) (several ng/ml) serve as highly active cofactors with selected polyanions for inducing generation of AK cells. The cofactors have been inactive or poorly active alone but have enhanced induction of AK cell activity by polyanions. For example, induction of AK cell activity by high molecular weight dextran sulfate (MW 500,000) has been enhanced at least 10 fold by 10 ng/mL of LPS purified from E. coli. With LPS, we routinely include indomethacin, which is inactive alone but blocks suppressive effects of prostaglandins on the generation of AK-cell activity. Dextran (MW 500,000) and relatively small molecules of dextran sulfate (MW 8,000) have been inactive even with LPS and indomethacin. Studies with recombinant cytokines indicate that the cofactors do not act simply by inducing release of IL-1, TGFα, or interferon, well-established cell products resulting from LPS stimulation. We speculate that variability in AK cell responses to polyanions, used without cofactors, may represent variability in background activation of ancillary cells that is enhanced and normalized in vitro by exogenous LPS-related factors.

This year we have also extended our work with AK cell populations that exhibit veto activity, that is, inactivation of foreign T cells which recognize antigens on the veto cell surface. We typically detect veto activity by adding strain B veto cells to an A against (B x C) F1 CML response in vitro

and by observing specific suppression of the A anti B response. We have found that optimal veto cell activity results from 1) preculturing thymocytes rather than splenocytes, 2) preculturing thymocytes with high doses of recombinant IL-2 (300-1000 U/ml), 3) preculturing 6-7 days, and 4) adding veto cells to an alloresponse on each of several days early in the course of the response.

Proposed Course of Project:

1. Retargeting the immune system against human tumor cells. In collaboration with Dr. David Segal we plan to optimize the anti-tumor effects of human leukocytes retargeted against human ovarian cancer cells in the nude mouse model. To this end we plan to 1) identify additional anti-tumor monoclonal antibodies which are effective in heterocrosslinked antibody preparations, 2) retarget leukocytes other than lymphocytes, such as neutrophils and monocytes, by using in the heterocrosslinked antibody preparations monoclonal antibodies against Fc receptors, and 3) optimally activate the cytotoxic activity of leukocytes prior to use with heterocrosslinked antibodies by exposing the leukocytes to cytokines (e.g. rIL-2, rIL-4, rIL-6, and rINFα) and pharmacologic agents which increase the intracellular cGMP/cAMP ratio. We also plan to study several issues which will bear directly on potential clinical protocols: 1) the size of tumor burden that can be effectively treated by retargeting, 2) whether retargeting will improve host survival, 3) why leukocytes from some donors are resistant to retargeting, 4) whether retargeting will result in anti-tumor activity against freshly explanted ovarian carcinoma cells, 5) whether host leukocytes can be recruited for anti-tumor activity by retargeted leukocytes, and 6) whether the most potent source of effector cells for retargeting (e.g. lymphocytes versus monocytes) varies between donors.

2. MHC-nonrestricted cytotoxicity of activated killer (AK) cells against tumors. We plan to investigate the following issues concerning AK cells with veto activity: 1) the phenotype of veto cells, 2) the mechanism of action of veto cells, and 3) whether veto cells will inhibit graft rejection in vivo. We also plan to address the following issues concerning generation of AK cells: 1) whether macromolecular dextran sulfate with LPS-related factors and indomethacin will induce AK cells in vivo in mice, and b) whether non-toxic LPS related molecules, such as Lipid X, will work as a cofactor with macromolecular polyanions for inducing AK cell activity. We speculate that the combination of macromolecular polyanions and non-toxic LPS related factors will be less toxic and more potent than IL-2 for generating AK cells in vivo.

Publications:

Titus JA, Garrido MA, Hecht TT, Winkler DF, Wunderlich JR, Segal DM. Human T cells targeted with anti-T3 cross-linked to antitumor antibody prevent tumor growth in nude mice. J Immunol 1987; 138:4018-4022.

Segal DM, Wunderlich JR. Targeting of cytotoxic cells with heterocrosslinked antibodies. Cancer Invest 1988;6:83-92.

Segal DM, Snider DP, Perez P, Qian J-H, Titus JA, Garrido MA, Winkler DA, Wunderlich JR. The use of heterocrosslinked monoclonal antibodies in target-

ing cytotoxic cells and in facilitating antigen presentation. Actua Sandoz 1988;in press.

Segal DM, Garrido MA, Perez P, Titus JA, Winkler DA, Ring DB, Kaubisch A, Wunderlich JR. Targeted cytotoxic cells as a novel form of cancer immunotherapy. Mol Immunol 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09251-18 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05018-17 I

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

Target Cell 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	EIB, NCI
Others:	W. Munger	Investigator	EIB, NCI
	K. Andrews	Microbiologist	EIB, NCI
	C.W. Reynolds	Investigator	BTB, FCRF, NCI
	H. Young	Expert	BTB, FCRF, NCI
	A. Kuta	Guest Researcher	EIB, NCI
	M. Kolber	Medical Staff Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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.)*

We have examined the ability of cytotoxic lymphocytes to killing of mammalian red blood cells as simple target cells. Using crosslinked hybrid antibodies against the T cell receptor and a target membrane antigen, efficient lysis of both red cell and nucleated targets could be achieved using cloned CTL. Splenic T cells or thymocytes do not cause detectable target lysis. Surprisingly, cloned helper T cells also killed red cell targets but not nucleated targets when triggered by this method. The physiology of red cell lysis by both effectors appears to be similar to classical CTL-mediated killing of nucleated targets. The mechanism of lysis by LGL granule cytolysin as been analyzed by taking advantage of the observation that both low pH and low ionic strength (in isotonic sucrose) block lysis at a step after cytolysin binding to target cells. After washing off all unbound cytolysin a stable intermediate is formed which will lyse when resuspended in a buffer containing isotonic saline at neutral pH. Calcium is required for this lytic step by either route of intermediate formation. The binding step requires calcium in low ionic strength, but not at low pH. The bound cytolysin can be digested off by proteases or neutralized by anti-cytolysin antibodies. When nucleated tumor cells are exposed to cytolysin at sub-lethal doses, they show evidence of membrane injury as indicated by their rapid membrane depolarization and loss of ions and small markers. However, this sublethal injury is repaired by the tumor cells within a few minutes, as shown by the restoration of membrane potential and normal distribution of sodium and potassium. We have found that polystyrene beads of diameter 3-15 mμ coated with both anti-T3 and anti-target antibody will trigger CTL to kill cells not normally recognized. This lysis appears to be a special case of bystander lysis, which is classically not observed in CTL cytotoxicity. We have developed a new general method for measurement of cytotoxicity using target labeling by the fluorescent dye BCECF. This should replace the standard ^{51}Cr release method for many purposes.

Project Description

Major findings:

We have studied the killing of mammalian red blood cells as simple target cells for CTL since these cells are unlikely to undergo a postulated "programmed cell death" or to have receptors for cytotoxic lymphokines. Using heteroconjugates, i.e., crosslinked hybrid antibodies against the T cell receptor and a red cell membrane antigen, efficient lysis of both red cell and nucleated targets could be achieved using cloned CTL. Splenic T cells or thymocytes do not cause detectable target lysis. Surprisingly, cloned helper T cells also killed red cell targets but not nucleated targets when triggered by heteroconjugates. These T helper clones do not contain detectable cytolysin in their granules, which may suggest that another lytic agent is responsible. The physiology of red cell lysis by both effectors appears to be similar to classical CTL-mediated killing of nucleated targets.

The mechanism of lysis by LGL granule cytolysin as been analyzed by taking advantage of the observation that low pH and low ionic strength (in isotonic sucrose) block lysis at a step after cytolysin binding to red cells. Thus we can wash off all unbound cytolysin and produce a stable intermediate which will hemolyse when resuspended in a buffer containing isotonic saline at neutral pH. Calcium is required for this lytic step by either route of intermediate formation. The binding step requires calcium in low ionic strength, but not at low pH. The bound cytolysin can be digested off by proteases or neutralized by anti-cytolysin antibodies. When nucleated tumor cells are exposed to cytolysin at sub-lethal doses, they show evidence of membrane injury as indicated by their rapid membrane depolarization and loss of ions and small markers. However, this sublethal injury is repaired by the tumor cells within a few minutes, as shown by the restoration of membrane potential and normal distribution of sodium and potassium. This injury requires $Ca^{+2}>10^{-5}M$, but tends to be inhibited by $Ca^{+2}>10^{-3}M$, by $Zn^{+2}>10^{-5}M$, or by pH <7.

We have found that polystyrene beads of diameter 3-15 mμ coated with both anti-T3 and anti-target antibody will trigger CTL to kill cells not normally recognized. Both antibodies must be on the same bead, and 3 mμ beads are the most efficient. This lysis appears to be a special case of bystander lysis, which is classically not observed in CTL cytotoxicity.

We have developed a new general method for measurement of cytotoxicity which should replace the standard 51Cr release method for many purposes. Our method utilizes the fluorescent dye BCECF, which is loaded into target cells as an ester. The dye retained by target cells after incubations of 4 hours or less with cytotoxic effectors is measured by a newly developed scanning microfluorimeter which reads a 96 well microtiter plate in about one minute. This technique is thus faster and cheaper than the 51Cr method, and does not involve radioactivity.

Proposed course: More detailed studies of red cell killing by CTL and T helper cells are being carried out, with the particular focus in examining the

question of the role of cytolysin. In particular, we will see if a prolonged "killer cell independent" lysis phase exists, as has been documented with nucleated targets. This would be hard to reconcile with a cytolysin-mediated injury, as cytolysin kills within a few minutes. We will check for the presence of cytolysin in T helper cells by fluorescence microscopy of fixed and premeabilized cells using anti-cytolysin antibody.

In order to extend our characterization of the-intermediates in cytolysin-mediated hemolysis, we would like to know how the bound cytolysin molecules are interacting with the red cells. In collaboration with Robert Dourmashkin we will examine the membrane surface by negative stain EM to look for any visible structures not on the normal membrane. If we can successfully label the cytolysin with ^{125}I, we will examine whether cytolysin requires detergent to solubilize it from the membrane (implying a membrane insertion), or if it can be liberated with chelators, chaotropic agents, or salts (implying no insertion into the bilayer). We will also see if the label has become aggregated when extracted with sodium deoxycholate or with SDS as we have shown for the fully assembled cytolysin complex in lysed red cells. In an extension of this project, we will look for similar intermediates with cytolysin-treated tumor cells. The primary motivation for this is to see if we can identify similar cytolysin-target intermediates created by CTL and LGL. If such intermediates are formed, we will see if they are neutralized by anti-cytolysin antibodies. A positive result in this experiment would directly implicate the cytolysin in the lethal hit delivered by CTL or LGL.

Publications:

Yue CC, Reynolds CW, Henkart PA. Inhibition of cytolysin activity in granules of large granular lymphocytes by lipids: Evidence for a membrane insertion mechanism of lysis. Mol Immunol 1987;24:647-653.

Heart P, Yue CC. The role of cytoplasmic granules in lymphocyte cytotoxicity. Prog Allergy 1988;40:82-110.

Kolber MA, Quinones RR, Gress RE, Henkart PA. Measurement of cytotoxicity by target cell release and retention of the fluorescent dye bis-carboxyethyl-carboxyfluroescein (BCECF). J Immunol Methods 1988;108:255-264.

Albright JW, Munger WE, Henkart PA, Albright JF. The toxicity of rat large granular lymphocyte tumor cells and their cytoplasmic granules for rodent and African trypanosomes. J Immunol 1988;140:2774-2777.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09252-17 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05033-16 I

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	EIB, NCI
Others:	S. A. Rosenberg	Chief	SB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	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

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.

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 09253-16 E

PERIOD COVERED
October 1, 1987 to September 30, 1988 formerly Z01 CB 05035-15 I

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Function of B Lymphocyte Fcγ Receptors

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

PI:	H. B. Dickler	Senior Investigator	EIB, NCI
Others:	G. Laszlo	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.0	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 goal of this project is to characterize the function of B lymphocyte Fcγ receptors. 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 utilizing antigen-antibody complexes 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 may be involved in generating the negative signal. Studies using monoclonal anti-FcγR antibody (2.4G2) in a variety of forms including native, chemically cross-linked into homodimers or heterodimers with anti-δ or $F(ab')_2$ anti-μ antibodies, and on a Sepharose matrix indicate that the monoclonal antibody only generates the negative regulatory signal if effective cross-linking of the receptor is obtained. Various B lymphocyte populations have been evaluated for susceptibility to Fcγ receptor mediated down-regulation. LyB5 negative B cells are susceptible but antigen-primed B cells are not. B cells from autoimmune MRL/l mice are susceptible but not those from autoimmune NZB mice. Lack of responsiveness to Fcγ receptor downregulation may play a pathogenic role in NZB autoimmune mice. IL-4 specifically induces a marked (90%) loss of binding of complexes to B lymphocyte FcγR II. The IL-4 induced loss of binding of complexes was time and temperature dependent and was reversible. IFN-γ could partially prevent this effect. The loss of binding of complexes did not appear to be due simply to a loss of expression of FcγR II because IL-4 induced only a moderate reduction in the binding of two FcγR II specific monoclonal antibodies.

Project Description

Major Findings: 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). This was true even under conditions where antigen receptor occupancy (in the absence of complexes) did not increase the magnitude of the response (i.e., the response itself was antigen-independent). Proliferation was also not affected in this system. Kinetic experiments revealed that both Fcγ receptors and antigen receptors had to be occupied simultaneously by their respective ligands during the early hours of the response in order to generate the downregulatory signal. Thus, the downregulatory signal is generated cooperatively by Fcγ receptors (surface IgM). The previously described interaction between these two receptors may play a role in the generation of the downregulatory signal.

Studies with monoclonal anti-Fcγ receptor antibodies (2.4G2) indicated that in native form they did not affect B lymphocyte responses. This was true over a broad dose range and when given simultaneously or 24 hours prior to B lymphocyte stimulation. Moreover, homodimers made with 2 different chemical cross-linkers also did not affect responses. Binding studies indicated that such preparations were cross-linking at least 4 FcγR. Attempts to produce large homomultimers resulted in inactivation of the 2.4G2. In contrast, 2.4G2 chemically cross-linked into heterodimers with $F(ab')_2$ anti-mu, or bound to a Sepharose matrix inhibited B lymphocyte responses in a fashion similar to antigen-antibody complexes. These studies indicate that monoclonal anti-FcγR antibodies only downregulate B lymphocyte responses if sufficient cross-linking of the receptor is obtained, and that internalization of the ligand is not required. These studies suggest that 2.4G2 can be used to regulate B lymphocyte responses if utilized in an appropriate form.

B lymphocytes from various sources have been assessed for their susceptibility to Fcγ receptor mediated downregulation. LyB5 negative B cells (from CBA/N mice) were susceptible, while antigen-primed B lymphocytes were not. These results are consistent with our earlier conclusion that early B lymphocytes are most susceptible to this down regulatory signal. B cells from autoimmune MRL/1 mice (which appear to have a T cell abnormality) were susceptible but B cells from autoimmune NZB mice (which appear to have a B cell abnormality) were not. The latter was true at any concentration of complexes and with NZB mice of any age. Thus, the lack of susceptibility to Fcγ receptor mediated downregulation may play a role in the hyperresponsiveness of NZB B lymphocytes.

B lymphocytes cultured for 24 h with recombinant IL-4 lost (90%) the capacity to bind IgG complexes as assessed by flow microfluorometry. Specificity was indicated by the observations that: IL-1, 1L-2, IL-3, GM-CSF, and IFN-γ did not inhibit the binding of complexes; IL-4 did not have a similar effect on other B lymphocyte membrane molecules; and inhibition was completely prevented by monoclonal anti-IL-4 (11B11). Inhibition was dose-dependent (>60% inhibition with 1 U/ml and maximal with $\geq$30 U/ml), time dependent (no effect at 4 h and maximal at 24 h), temperature dependent (no effect at 4^{o}C), and reversible (cells which had lost the capacity to bind complexes due to IL-4 regained this ability when recultured for 24 h in the absence of IL-4). The inability to bind complexes could not be accounted for solely by a loss of expression of FcγR II because IL-4 induced only a moderate reduction in the binding of two FcγR II specific monoclonal antibodies (20% for 2.4G2 and 32% for K9.361). These results suggest that IL-4 regulates the binding of complexes to B lymphocyte FcγR by inducing some form of alteration of the receptor. This effect could play a role in the regulation of B lymphocyte responses by preventing FcγR II mediated downregulation.

Publications:

Dickler HB, Kubicek MT. Effects of various form of monoclonal anti-FcγR II (2.4G2) on B lymphocyte responses. Mol Immunol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09254-14 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05050-13 I

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

The Manipulation of Immune Processes with Heterocrosslinked Antibodies

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

PI:	D. M. Segal	Senior Investigator	EIB, NCI
Others:	D. P. Snider	Visiting Fellow	EIB, NCI
	Q. Jia-Hua	Guest Researcher	EIB, NCI
	J. Wunderlich	Senior Investigator	EIB, NCI
	M. Garrido	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	4.5	1.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

1. Targeted T cell cytotoxicity resides in the low density fraction of PBL. Activity can be generated in the high density fraction by treatment with anti-CD3 antibody and IL-2.

2. Targeted tumoristatic activity has been detected in a long term in vitro tumor growth assay. This activity resides in the $T4^+$ and $T8^+$ subsets of T cells.

3. Human T cells and K cells targeted with anti-T3 and anti-FcR, heterocrosslinked to anti-tumor antibodies eradicate established human ovarian cancer cells in nude mice.

4. Heterocrosslinked antibodies containing an antibody against a soluble antigen linked to an antibody against a cell surface determinant on an antigen presenting cell (APC), greatly enhance the efficiency of antigen presentation. Enhanced presentation has been seen when antigen was targeted to Fc receptors, surface immunoglobulin, MHC class I and MHC class II molecules on the APC. Targeted antigen presentation is antigen specific and I-A restricted.

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

Project Description

Major Findings:

1. Targeted Cytotoxicity: In Vitro Studies

We have shown that cytotoxic cells can be induced to lyse cells other than their natural targets with antibody heteroconjugates which contain an antibody against the cytotoxic cell receptor (CCR) crosslinked to an antibody against a target cell structure, for example a tumor antigen. By linking the target cell directly to the CCR, the heterocrosslinked antibodies promote target cell lysis. We are now focusing on methods of identifying and generating subsets of cells from human peripheral blood which can be targeted against pathogenic cells for potential therapeutic purposes. We have established that all of the cytotoxic activity in normal peripheral blood T cells induced by anti-CD3 containing heteroconjugates resides in the CD8+,CD4- subset. Recently we have found that this activity can further be localized to the light fraction on a Percoll density gradient, consisting of CD3+, CD8+, Leu-7+ cells. These cells are extremely potent cytolytic effectors since they comprise less than 5% of the lymphocytes. Although the cytolytic activity of these cells is enhanced by IL-2, we have not yet found a way of inducing their proliferation. Therefore, the total amount of cytolytic activity in the low density cells remains low after IL-2 stimulation. The vast majority of T cells reside in the Percoll dense fraction. These cells have no cytolytic activity on day 0 or after 1-2 days stimulation with IL-2. However, 5 days of stimulation with IL-2 plus anti-CD3 bound to the floor of the tissue culture flask, produces both proliferation and the generation of targetable cytolytic effector cells.

We have also demonstrated that the targetable K cells reside in a low density fraction of PBL, and are $Fc_\gamma RIII+$, Leu7+, CD3-. This population cannot be expanded with IL-2 or anti-$Fc_\gamma RIII$, although cytotoxicity is readily increased by IL-2. In addition to $Fc_\gamma RIII$, two other $Fc_\gamma R$ have been identified in the human. $Fc_\gamma RI$ is a high affinity receptor for IgG1 on monocytes, and $Fc_\gamma RII$ is a low affinity receptor on monocytes and neutrophils. Monoclonal antibodies against $Fc_\gamma RI$ and II have been developed in the laboratories of Drs. Clark Anderson and Michael Fanger. Recently we have entered into a collaboration with Michael Fanger, and have prepared heteroconjugates containing anti-$Fc_\gamma RI$ and anti-$Fc_\gamma RII$. Both of these heteroconjugates target neutrophils and monocytes against tumor cells, and cytotoxicity is enhanced by incubating the cells with γ-interferon, but not IL-2.

Our first in vivo studies employed classic Winn-type tumor neutralization assays, using the LS174T human colon adenocarcinoma line, which forms tumors when injected subcutaneously in nude mice. In these studies targeted T and K cells were both able to prevent the establishment and growth of the tumors at low effector:target ratios (eg at 1:1 or less). Because all of the targeted T cell cytotoxicity as measured in a ^{51}Cr-release assay resided in the CD8+, CD4- subset, we expected the tumor neutralizing activity to be in this subset

as well. However we found that most of the tumor neutralizing activity was in the CD4+ subset, with some activity remaining in the CD8+ cells. It was clear from these studies that tumor neutralization and ^{51}Cr release measured two different activities. In view of these results we have established an in vitro tumoristasis assay in order to study the mechanisms of tumor neutralization. In our in vitro tumoristasis assays, tumor cells (LS174T human colon adenocarcinoma line) are incubated several days with or without PBL targeted with anti-CD3 x anti-tumor. At the end of the experiment, PBL are washed away (LS174T is adherent) and the remaining tumor cells are counted. Results from these studies show that targeted PBL exhibit strong tumoristatic activity, and that much of this activity resides in the CD4+ subset. Preliminary evidence suggests that targeted tumoristasis may be mediated through a soluble factor.

2. Targeted Cytotoxicity: In Vivo Studies

We have recently asked whether targeted cytotoxic cells might be able to eradicate an established tumor. In these studies we have treated intraperitoneal growth of the human ovarian adenocarcinoma line, OVCAR3, with intraperitoneal injections of targeted PBL. We chose this system because both the tumor and the treatment are confined to the peritoneum, thereby minimizing problems of delivering the targeted cytotoxic cells to the tumor. Since OVCAR3 grows ip in nude mice in much the same way that ovarian carcinoma grows in female cancer patients, these studies could form the basis for initiating clinical trials, using targeted PBL to treat ovarian cancer. Nude mice are given tumor cells ($5x10^6$ - $1x10^7$ per mouse) on day 0 and are treated with four doses of PBL alone or PBL plus heteroconjugate on days 5 and 6, 10^7 cells per dose at 12 hr intervals. They are then sacrificed on day 15 and tested for tumor growth by ip lavage. Positive mice give a large tumor cell pellet upon centrifugation of the peritoneal wash, while negative mice have no detectable tumor cells. Using this procedure we have seen highly significant protection against tumor growth by targeted T cells. For example, in 12 different experiments, of 75 mice given tumor plus untreated PBL, 76% were tumor positive at day 15. By contrast, of 88 mice given tumor plus PBL coated with anti-CD3 crosslinked to the anti-tumor antibody, 113F1 (536), only 39% were tumor positive at the end of the experiment. The difference between these two groups is significant to $P<.001$. Interestingly, in 3 out of 12 experiments, PBL treated with heteroconjugate gave no protection, while in the other nine experiments strong protection was observed; only 28% of 69 mice were tumor positive at the end of the experiment in the group showing significant protection. Thus targeted PBL from many donors exhibit a strong anti-tumor effect in this system, while PBL from some other donors show no effect. Using this short term assay, we have identified, to date, 3 different anti-tumor mAb which can be used in heteroconjugates to successfully treat OVCAR3. We have also found that mice given heteroconjugate but no PBL, PBL with an irrelevant heteroconjugate (anti-HLA class I x 113F1 or anti-CD3 x anti-DNP), or an unconjugated mixture of anti-CD3 and 113F1, were not protected against tumor growth.

3. Targeted Antigen Presentation

T helper cells recognize antigen in a degraded form, bound to MHC class II molecules on the surfaces of APC. Non-specific APC take up antigen by

fluid phase pinocytosis, a relatively inefficient process requiring high antigen concentrations. The pinocytosed antigen is then degraded and returned to the surface in association with class II molecules. We have shown that non-specific APC can be rendered specific by targeting antigen to their surfaces with heterocrosslinked antibodies. Thus heteroconjugate-coated APC can present antigen to T cells at concentrations which would normally be too low to elicit a response. The presentation of antigen to T cells can lead to the expansion and activation of antigen-specific T-helper clones, which in turn are essential for the generation of cellular and humoral responses against that antigen.

In order to measure antigen presentation we have used two murine T helper hybridomas, A22.E10, [I-A^d restricted, ovalbumin (OVA) specific] and C10.9 [I-A^k restricted, hen egg lysozyme (HEL) specific]. Stimulation of lymphokine production by these hybridomas was used to measure antigen presentation. Lymphokine was assayed by measuring proliferation of the lymphokine-dependent CTLL line. TNP-OVA was targeted to APC using heteroconjugates containing anti-DNP, and HEL was targeted using 3 different anti-HEL mAbs obtained from Dr Sandra Smith-Gill. These antibodies were crosslinked to mAbs against MHC class I and class II antigens, surface immunoglobulin, or $Fc_\gamma R$. By using the TA3 B cell hybridoma as APC, we found that antigen targeted to both class I and class II MHC antigens was presented 500-1000 times more efficiently than untargeted antigen, and that targeting to surface immunoglobulin and $Fc_\gamma R$ also improved the efficiency of presentation, but to a lesser degree. Specificity controls showed that antigen targeting required both antibodies of a heteroconjugate to be linked to one another; either antibody alone, or a mixture of the two antibodies did not result in enhanced antigen presentation. Moreover, one antibody within a heteroconjugate had to be specific for the antigen while the other had to be specific for a component on the APC surface. In all cases, targeted APCs were recognized by the T helper hybridomas with the same MHC restriction and antigen specificity as an APC generated using high concentrations of antigen and no heteroconjugate. Enhanced presentation titered out at heteroconjugate concentrations of 10-100 ng/ml.

Splenic B cells and irradiated adherent cells (presumably macrophages) were also tested as targeted APC. With B cells, heteroconjugates containing anti-sIg, anti-class I and anti-class II, but not anti-$Fc_\gamma R$ gave enhanced presentation, whereas effective antigen targeting to the adherent cells was seen only when antigen was targeted to class I or class II molecules.

B cells which have been coated with heteroconjugate and antigen, and washed, present that antigen to the T helper hybridomas. We have taken advantage of this observation in order to follow the processing and presentation of HEL to C10.9 cells. As expected, presentation of targeted antigen does require a time dependent processing step. B cells containing a single coat of antigen were incubated at 37° for varying periods of time, and were either treated with pronase, to remove surface-bound antigen, or were fixed with paraformaldehyde, to stop processing. Cells fixed after 4 hr incubation were unable to present antigen. However B cells fixed after 10 hr incubation gave maximal presentation when antigen had been targeted to surface IgD. By

contrast, when antigen was targeted to class I or class II molecules, maximal presentation by these B cells occurred after 18 hr incubation. Antigen targeted to surface immunoglobulin was also endocytosed more rapidly than that targeted to MHC molecules as judged by pronase stripping. Thus Ig-targeted antigen was presented if the cells were stripped after 1 hr incubation, whereas MHC-targeted antigen required much longer incubation times before presentation became resistant to stripping.

We have directly measured the endocytosis of antigen bound by heteroconjugates to B cell surfaces, using either fluorescent or radiolabeled antigen. As predicted from the presentation studies, antigen bound by heteroconjugates to surface immunoglobulin is endocytosed much more rapidly than antigen bound to MHC structures.

Preliminary studies have shown that heteroconjugates can aid in the development of antibody responses in vivo. Mice were injected with low doses of antigen (HEL) subcutaneously, in the presence or absence of heteroconjugates. After 2 weeks they were tested for serum antibody and were also given a second, low dose of antigen, in the absence of heteroconjugate. Two weeks later, they were again tested for antibody production. We have found that after the primary immunization no antibody could be detected in the serum, whether or not heteroconjugate was used. However after the second challenge, antibody was observed only in those animals which were primed with antigen plus heteroconjugate. Heteroconjugates containing anti-class I or anti-class II, but not anti-IgD antibodies were able to enhance priming, suggesting that the enhancement was not occurring via B cells. Interestingly, in some mouse strains, but not in others, heteroconjugates containing anti-$Fc_{\gamma}R$ also enhanced priming. Controls containing a mixture of the non-conjugated antibodies, or either antibody of the heteroconjugate alone did not enhance priming.

Proposed Course of Project

1. Ovarian Cancer Project.

Our current goal is to optimize conditions for eradicating ip ovarian tumor in nude mice. There are several aspects of this problem which we will investigate. 1. We will test heteroconjugates containing several different anti-tumor antibodies to find those which work best. 2. We will try to find better ways of activating PBL in order to get increased anti-tumor activities from all donors. 3. We will compare anti-tumor activities of targeted T cells with those of the K cells in the ovarian model, and then test whether monocytes or neutrophils, targeted with either anti-$Fc_{\gamma}RI$ or II might also be able to eradicate established tumors. 4. We will vary the treatment schedules, with a special interest in determining how long treatment can be delayed without loss of anti-tumor activity. 5. We will follow survival and tumor growth in long term experiments.

The ultimate goal of these studies is to aid in the development of clinical protocols in which ovarian cancer patients will be treated with

autologous targeted PBL, monocytes or neutrophils. Toward this end, we are collaborating with Dr. David McKean and coworkers at the Mayo Clinic, who are testing our heteroconjugates against fresh tumor cells from ovarian cancer patients. Once we have identified the best heteroconjugates (ie those giving the highest amounts of lysis against fresh tumor cells from a high percentage of patients in vitro), both Cetus and Centocor have agreed to produce hetero-crosslinked antibodies under conditions suitable for use in patients.

The next phase of these studies will be to develop in vivo models of tumors which can be treated by systemic administration of targeted lymphocytes.

2. Targeted Antigen Presentation.

Because a single coat of targeted antigen is efficiently presented to T helper cells, we are hoping that we will be able to follow the course of the antigen within the APC without extraneous noise from irrelevant antigen. In particular we will follow acidification of FITC-conjugated antigen by fluorescence quenching to determine when, and how much of the antigen enters acidic compartments, and we will also follow the degredation of radiolabeled HEL by SDS-PAGE. We have established a collaboration with electron microscopists, Drs. Leo Ginsel and Carolina Jost, of the University of Leiden, who have considerable experience following the intracellular trafficking of FcγR. We have several mAb to different sites on HEL, and we are planning to label targeted antigen at various stages of internalization with gold-antibody conjugates, using different anti-HEL antibodies in the hetero and gold conjugates. Other in vitro studies will look at the effects of cell surface polymerization on antigen presentation.

We are continuing with our in vivo studies of antigen presentation. Experiments will be done to maximize the enhancement of presentation by heteroconjugates. Different routes and schedules of immunization will be tested, and other heteroconjugates and mouse strains will also be used. If surface aggregation enhances presentation in vitro, we will also test it in vivo. As mentioned above, heteroconjugates can aid in the priming of an antibody response, but we also plan to follow their effects, if any, upon antigen-specific T cell proliferation. An ultimate goal of this project is to target tumor antigens to APC.

Publications

Leo O, Foo M, Henkart PA, Perez P, Shinohara N, Segal DM, Bluestone JA. Role of accessory molecules in signal transduction of CTL by anti-T cell receptor and anti-Ly-6.2C monoclonal antibodies. J Immunol 1987;139:3556-3563.

Titus JA, Perez P, Kaubisch A, Garrido MA, Segal DM. Human K/NK cells targeted with heterocrosslinked antibodies specifically lyse tumor cells in vitro and prevent tumor growth in vivo. J Immunol 1987;139:3153-3158.

Segal DM, Titus JA, Stephany DA. Fluorescence flow cytometry in the study of lymphoid cell receptors. Methods Enzymol 1987;150:478-492.

Leo O, Foo M, Segal DM, Shevach E, Bluestone JA. Activation of murine T lymphocytes with monoclonal antibodies: Detection on Lyt2+ cells of an antigen not associated with the T cell receptor complex but involved in T cell activation. J Immunol 1987;139:1214-1222.

Titus JA, Garrido MA, Hecht TT, Winkler DF, Wunderlich JR, Segal DM. Human T cells targeted with anti-T3 crosslinked to anti-tumor antibody prevent tumor growth in nude mice. J Immunol 1987;138:4018-4022.

Snider DP, Segal DM. Targeted antigen presentation using crosslinked antibody heteroaggregates. J Immunol 1987;139:1609-1616.

Segal DM, Wunderlich JR. Targeting of cytotoxic cells with heterocrosslinked antibodies. Cancer Invest 1988;6:83-92.

Segal DM, Garrido MA, Perez P, Titus JA, Winkler DA, Ring DB, Kaubisch A, Wunderlich JR. Targeted cytotoxic cells as a novel form of cancer immunotherapy. Mol Immunol 1988;in press.

Segal DM, Snider DP, Perez P, Qian J-H, Titus JA, Garrido MA, Winkler DA, Wunderlich JR. The use of heterocrosslinked monoclonal antibodies in targeting cytotoxic cells and in facilitating antigen presentation. Actua Sandoz 1988; in press.

Patent

David M. Segal and Pilar Perez. Target Specific Cross-Linked Heteroantibodies. U.S. Patent 6-778,670.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09255-13 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05062-12 I

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

Application of Flow Cytometry 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	EIB, NCI
	S.O. Sharrow	Chemist	EIB, NCI
Others:	J.P. Henrich	Microbiologist	EIB, NCI
	I.R. Uppenkamp	Visiting Associate	EIB, NCI
	Members of the Experimental Immunology Branch, NCI and Members of the Immunology Branch, NCI (see text)		

COOPERATING UNITS *(if any)* A. Schultz, CSL, DCRT; L. Barden, CSL; DCRT; J. Bluestone, U. of Chicago; D. DeLuca, USC; T.A. Waldmann, MB, DCBD, NCI; M.T. Lotze, SB, DCT, NCI; S.A. Rosenberg, SB, DCT, NCI; J.D. Shanley, VA Med. Ctr., U. of CT; D.H. Sachs, IB, DCBD, NCI; S.I. Katz, Dermatology Br., DCBD, NCI; R. Klausner, NICHHD.

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.1	1.1	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.)*

The Experimental Immunology Branch flow cytometry laboratory currently supports 60 individual research projects for 45 investigators. These investigations involve quantitative single cell analysis of parameters associated with cell types freshly prepared from different species/tissues, as well as spectrum of in vitro cultured cells. Basic research support is provided to members of the EIB and the Immunology Branch, as well as to other members of DCBD. Currently supported projects include a broad range of applications in the areas of cellular immunology, immunochemistry, transplantation biology, clinical immunology, immunogenetics, tumor immunology, and molecular immunology. These include, but are not limited to, the following studies: a) in vivo and in vitro analyses of intra-cellular signalling via T cell surface molecules, b) characterization of T cells from animals with genetic or induced immune dysfunction; c) studies of the pathogenesis of graft-versus-host disease; d) analyses of the correlations between T cell memory and the quantitative expression of cell surface adhesion molecules; e) investigations of T cell ontogeny and differentiation; and f) analyses of the mechanisms involved in marrow graft rejection versus acceptance.

GPO 914-918

Project Description

Major Findings: This report summarizes findings of eight project areas, emphasizing those aspects most heavily supported by the use of flow cytometry analysis.

Dr. A. Singer and colleagues utilized flow cytometry analysis in a series of investigations involving in vivo and in vitro analyses of intra-cellular signalling via T cell accessory molecules. In one of these studies, the protein kinase C activator PMA was used to treat freshly isolated murine spleen T cells and thymocytes for brief (15') periods at 37 degrees. It was found that peripheral T cells treated in this fashion exhibited, as expected, reduced surface expression of the T cell receptor complex. It was also found that CD4 expression on both mature peripheral $CD4^+8^-$ T cells and non-mature $CD4^+8^+$ thymocytes was down-modulated. In contrast, CD8 cell surface expression was not affected on either of these cell types. However, both CD4 and CD8 could be down-modulated on peripheral T cells using antibody-mediated cross-linking. These observations have implications for our understanding of the mechanisms by which accessory molecules contribute to T cell maturation and activation.

Dr. Shaw and colleagues have utlilized flow cytometry in extending their characterization of T cell expression of adhesion molecules. They have found that the amount of surface LFA-3 on human peripheral blood T cells correlates with functional capacity in that naive cells express low levels, while memory T cells express moderate amounts. Further, they have shown that quantitative LFA-3 expression correlates with that of CD2, LFA-1, 4B4, UCHL1 and Pgp-1 and inversely correlates (as does expression of the 5 molecules just listed) with that of 2H4. These findings have led to reinterpretation of studies which originally described the subset distributions of these antigens, and have led to a more thorough understanding of T cell maturation.

Dr. Segal and colleagues have received flow cytometry support in a series of investigations aimed at developing re-targeting methods of enhancing immune responses. In one of these studies it has been found that anti-immunoglobulin:anti-antigen cross-linked antibodies can enhance the antigen-presenting capacity of murine B cells. Flow cytometry is utilized in these studies not only for quantitative characterization of binding of the cross-links to B cells, but also for characterization of the kinetics and mechanisms of internalization of antigen:cross-linked antibody complexes.

Dr. Shearer and colleagues utilized flow cytometry analysis in their studies which analyze the pathogenesis of events associated with graft-versus-host (GVH) disease. They have shown that the effects of the GVH reaction of the host's immune system depend upon the class of MHC antigen recognized by the donor cells. They have further found that chimerism in these GVH animals reflects these cellular mechanisms of GVH-mediated damage. Thus a direct correlation was observed between the cell types required for GVH induction and the parental T cell phenotypes detected in the spleens of the GVH mice. In addition, this group has found that GVH-induced damage includes a defect in

the ability of the thymus to support the differentiation of functionally competent $CD4^+$ T cells. Since $CD4^+$ T cells bearing T-cell receptor are produced by GVH-damaged thymuses, and because functional capacity is restored by normal thymus grafts, this observation provides a unique model for analysis of T cell differentiation, as well as for characterization of immune dysfunction induced by GVH disease.

Dr. Sachs and colleagues (IB) have used flow cytometry analysis to phenotypically characterize and kinetically monitor T cell subset reconstitution in studies of allogeneic bone marrow transplantation in irradiated animals. These studies use mixtures of allogeneic marrow containing T cells and syngeneic T-cell-depleted marrow. In both swine and murine mixed bone marrow studies, it was found that high frequencies (20-50%) of $CD8^+$ allogeneic (to host) donor cells are detected in peripheral blood 2 to 3 weeks post reconstitution. These high frequencies of CD8+ T cells early after transplantation of allogeneic bone marrow containing mature T cells are consistent with graft versus host reactions and coincide with clinical symptoms of GVH. Following this acute phase, frequencies of allogeneic donor $CD8^+$ T cells decline, as do acute clinical symptoms. These data suggest that it may be possible to moderate GVH-induced immune dysfunction in bone marrow transplantation by mechanisms involving autologous cellular elements.

Dr. Waldmann (Metabolism Branch) and colleagues have received flow cytometry support for their studies of the therapeutic effects of anti-human Il-2 receptor antibody (anti-Tac) in adult T-cell leukemia (ATL), in which tumor cells are Tac^+. These studies demonstrated anti-tumor effects in 3 of 9 ATL patients treated. Flow cytometry analysis was used: a) to monitor injected mouse antibody on the surface of leukemic cells; b) to assist in evaluation of remission/relapse status of these patients; and c) for phenotypic characterization of circulating normal and leukemic cells.

S. Sharrow in collaboration with Dr. D. DeLuca (Medical University of South Carolina) and Dr. D. Sachs (IB) has analyzed the correlation between murine thymocyte differentiation and the cell surface expression of the MHC Class I antigen, Qa-2. While all peripheral T cells are $Qa\text{-}2^+$, only 5% of thymoctes express cell surface Qa-2. These studies demonstrated that Qa-2 was also expressed by T cells which differentiated in fetal thymus organ cultures. Analysis of normal adult thymocytes, using 3-color immunofluorescence and flow cytometry, showed that $CD4^+8^+$ "cortical" phenotype thymocytes did not express Qa-2. In contrast, subsets of CD3-bright, phenotypically mature thymocytes did express Qa-2. All $Qa\text{-}2^+$ cells expressed high levels of T-cell receptor (CD3-bright). These data demonstrate that Qa-2 expression uniquely identifies subsets of fully differentiated thymocytes. Further, these data suggest that an additional differentiation step, subsequent to upregulation of T-cell receptor expression, is required for conversion to the peripheral, $Qa\text{-}2^+$, functional phenotype.

S. Sharrow and Luther Barden (CSL, DCRT) have evaluated the application of automated cluster analysis techniques to multiparameter-flow cytometry data. It has been shown that: a) these techniques can identify subpopulations of low (<0.5%) frequency in heterogeneous populations containing large (>20)

numbers of subpopulations; b) the capacity of these techniques to provide data which permits simultaneous display of 4 or more parameters simultaneously is important in correct interpretation of complex data; and c) the technology provides increased efficiency of data analysis, when compared to conventional techniques. Based upon these results, a project has been implemented to construct a user-friendly list mode analysis work station for on-site installation in the EIB flow cytometry laboratory.

Publications:

Via CS, Sharrow SO, Shearer GM. Role of cytotoxic T lymphocytes in the prevention of lupus-like disease occurring in a murine model of graft-vs-host disease. J Immunolog 1987;139:1840-1839.

Shimada S, Caughman SW, Sharrow SO, Stephany D, Katz SI. Enhanced antigen-presenting capacity of cultured Langerhans cells is associated with markedly increased expression of Ia antigen. J Immunol 1987;139:2551-2555.

Lotze MT, Custer MC, Sharrow SO, Rubin LA, Nelson DL, Rosenberg SA. In vivo administration of purified human interleukin-2 to patients with cancer: development of interleukin-2 receptor positive cells and circulating soluble interleukin-2 receptors following interleukin-2 administration. Cancer Res 1987;47:2188-2195.

Mathieson BJ, Sharrow SO. Cell-surface differentiation antigens expressed on thymocytes and T cells of the mouse. In Miyasaka M, Trnka A. eds. Differentiation antigens in lymphohemopoietic tissues. New York: Marcel Dekker, 1988;65-151.

Shanley JD, Via CS, Sharrow SO, Shearer GM. Interstitial pneumonitis during murine cytomegalovirus infection and graft-vs-host reaction: characterization of bronchoalveolar lavage cells. Transplantation 1987;44:658-662.

Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, Shaw S. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. J Immunol 1988;140:1401-1407.

Moser M, Sharrow SO, Shearer GM. Role of $L3T4^+$ and $Lyt2^+$ donor cells in graft-vs-host immune deficiency induced across a class I, class II or whole H-2 difference. J Immunol 1988;140:2600-2608.

Segal DM, Titus JA, Stephany DA. The use of fluorescence flow cytometry for the study of lymphoid cell receptors. Methods in Enzymol 1987;150:478-492.

Snider DP, Segal DM. Targeted antigen presentation using crosslinked antibody heteroaggregates. J Immunol 1987;139:1609-1616.

Parent L, Matis L, and Singer D. Enhanced expression of class I MHC by ethanol. FASEB Journal 1987;1:469-473.

Lucas P, Gress RE. Specific selection of lymphocyte subpopulations by avidin-biotin immune rosetting. J Immunol Methods 1987;99:185-190.

Gress R, Moses R, Suzuki T, Lowman M, Sakamoto K, Pennington L, Sachs DH. Biparental bone marrow transplantation as a means of tolerance induction. Trans Proc 1987;19:95-97.

Lucas P, Quinones RD, Moses RD, Nakamura H, Gress RE. Alternative donor sources in HLA-mismatched marrow transplantation: T cell depletion of surgically resected cadaveric marrow. Bone Marrow Transplant 1988;3:211-220.

Leo O, Foo M, Segal DM, Shevach E, Bluestone JA. Activation of murine T lymphocytes with monoclonal antibodies; detection on $Lyt2^+$ cells of an antigen not associated with the T cell receptor complex but involved in T cell activation. J Immunol 1987;139:1214-1222.

Matis LA, Cron R, Bluestone JA. Major histocompatibility complex-linked specificity of gamma delta receptor-bearing T lymphocytes. Nature 1987;330: 262-264.

Hirsch R, Eckhaus M, Auchincloss Jr. H, Sachs DH, Bluestone JA. Effects of in vivo administration of anti-T3 monoclonal antibody on T cell function in mice. I. Immunosuppression of transplantation responses. J Immunol 1988;140:3766-3772.

Kaldjian E, McCarthy SA, Sharrow SO, Littman DR, Klausner RD, Singer A. Non-equivalent responses of L3T4 and Lyt2 to protein kinase C activation by PMA. FASEB Journal, in press.

Mizuochi T, Tentori L, Sharrow SO, Kruisbeek AM, Singer A. Differentiation of Ia-reactive $CD8^+$ murine T cells does not require Ia engagement. Implications for the role of CD4 and CD8 accessory molecules in selection of the developing T cell repertoire. J Exp Med, in press.

Kolber MA, Walls RM, Hinners ML, Singer DS. Evidence of increased Class I MHC expression on human peripheral blood lymphocytes during acute ethanol intoxication. Alcoholism: Clinical and Experimental Research, in press.

Fukuzawa M, Via CS, Shearer GM. Defective thymic education of $L3T4^+$ T helper cell function in graft-vs-host mice. J Immunol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09256-01 E

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

In Vitro Study of MHC-Specific T Cells

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

PI:	A. Singer	Senior Investigator	EIB, NCI
Others:	T. Mizuochi	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.15	1.15	

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 has been aimed at identifying and characterizing the T cell subsets that initiate and effect anti-MHC responses in vitro. We have identified and characterized 3 distinct T helper cell populations that function in vitro to initiate anti-MHC responses, and have studied 3 distinct T killer cell populations that function in vitro as anti-MHC effector cells. We have characterized these cell populations with regard to their specificity, phenotype, activation requirements, and differentiation requirements.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Major Findings:

In contrast to conventional immune responses against foreign protein antigens that involve a single population of Th cells, we have characterized 3 distinct populations of Th cells that function to initiate anti-MHC responses in vitro, based on their recognition of different MHC specificities expressed on the surface of accessory (Acc) cells: (a) one Th cell population recognizes class II MHC allodeterminants, (b) one Th cell population recognizes class I MHC allodeterminants, and (c) one Th cell population recognizes an antigenic complex composed of foreign class I and self-class II MHC determinants. The two class II restricted Th cell populations are phenotypically $CD4^+$, whereas the class I restricted Th cell population is phenotypically $CD8^+$. In cell mixing experiments, each of these 3 Th cell populations collaborated with distinct populations of helper-dependent Tk cells, and so fulfilled the conventional definition of a Th cell. One common function performed by each of the 3 anti-MHC Th cell populations was the secretion of IL-2. Indeed, triggering Th cells to secrete IL-2 is a necessary event for the initiation of anti-MHC Tk cell responses, since Tk cell precursors require IL-2 for their activation and clonal expansion as indicated by the ability of anti-IL-2 receptor mAb to block Th cell activation of anti-MHC Tk cells.

To begin analyzing the basis for the disparity between the limited response repertoire expressed by IL-2 secreting $CD8^+$ Th cells and the wide response repertoire expressed by IL-2 dependent $CD8^+$ Tk cells, we examined the influence of thymic MHC determinants on the specificity of these two functionally distinct $CD8^+$ T cell populations. We engrafted $H\text{-}2^b$ nude mice with thymic lobes expressing MHC class I alloantigens, so that the thymus was the only site in the animal expressing the MHC class I alloantigen. We found that isolated expression of class I MHC alloantigens in the thymus during T cell ontogeny results in the specific deletion of IL-2 secreting $CD8^+$ Th cells against that class I alloantigen, but does not delete $CD8^+$ Tk cells against that class I alloantigen. Thus, by engrafting nude mice with allogeneic thymic lobes, we could experimentally re-create for any class I alloantigen the functional disparity between $CD8^+$ Th and Tk cells that we observed in normal mice against selected class I allodeterminants, such as K^{bm6}.

Publications:

Golding H, Mizuochi T, McCarthy SA, Cleveland CA, Singer A. Relationship among function, phenotype, and specificity in primary allospecific T cell populations. Identification of phenotypically identical but functionally distinct primary T cell subsets that differ in their recognition of MHC class I and class II allodeterminants. J Immunol 1987;138:10-17.

Moser M, Mizuochi T, Sharrow SO, Singer A, Shearer GM. Graft-versus-host reaction limited to a class II MHC difference results in a selective deficiency in $L3T4^+$ but not $Lyt2^+$ T helper cell function. J Immunol 1987;138:1355-1362.

Singer A, Munitz TI, Golding H, Rosenberg AS, Mizuochi T. Recognition requirements for the activation, differentiation, and function of T helper cells specific for class I MHC alloantigens. Immunol Rev 1987;98:143-170.

Mizuochi T, Singer A. Intrathymic differentiation and tolerance induction of lymphokine secreting $Lyt2^+$ T helper cells. J Immunol 1988;140:1421-1424.

McCluskey J, Munitz TI, Boyd L, Germain RN, Coligan JE, Singer A, Margulies DH. Cell surface expression of the amino-terminal domain of A_α^k: Recognition of an isolated major histocompatibility complex antigenic structure by allo-specific T cells but not alloantibodies. J Immunol 1988;140:2081-2089.

Shearer GM, Singer A, Mizuochi T, Buller M, Hugin A, Morse HC. Importance of $CD8^+$ T helper cell function in AIDS. J Inf Dis 1988;in press.

Mizuochi T, McKean DJ, Singer A. Interleukin-1 as a cofactor for lymphokine secreting $CD8^+$ murine T cells. J Immunol 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09257-13 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05067-12 I

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

Mechanisms of Cellular Immune Responses

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

PI:	S. Shaw	Senior Investigator	EIB, NCI
Others:	W. Makgoba	Fogarty Visiting Associate	EIB, NCI
	M. Sanders	Medical Staff Fellow	EIB, NCI
	G. Ginther-Luce	Chemist	EIB, NCI
	Y. Shimizu	IRTA	EIB, NCI
	K. Horgan	Fogarty Visiting Associate	EIB, NCI
	G. van Seventer	Fogarty Visiting Associate	EIB, NCI
	R. Siraganian	Section Chief	LMI, IRP, NIDR

COOPERATING UNITS *(if any)*

T. Springer, M. Dustin, Dana Farber Cancer Institute, Boston, MA; P. Mannoni, Institute J. Paoli-I. Calmette, Marseille, France; E. Clark, U. Washington, Seattle WA

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.2	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.)*

Our studies continue to be directed at elucidating the mechanisms of T cell recognition, with particular emphasis on antigen-independent T cell adhesion as a critical early step in the process of recognition. Major progress has been in understanding the functional role of the ICAM-1 molecule which our previous functional studies have suggested functions as a ligand for LFA-1. ICAM-1 has been purified by immunoaffinity chromatography. Each of two sites on ICAM-1 defined by monoclonal antibodies are implicated in its adhesion function. Furthermore, ICAM-1 must be an adhesion ligand per se since ICAM-1 immobilized on plastic mediates LFA-1 dependent adhesion of T, B and myeloid cells. This simplified system of cell binding to ICAM-1 allows isolation/analysis in a well defined system of important features of more complex cell interactions including characteristic divalent cation requirements, and a dissociation phase that occurs following the binding phase.
Recent studies confirm and extend our previous understanding that human naive and memory cells are distinguished phenotypically by differences in expression not only of adhesion molecules CD2, LFA-3 and LFA-1, but also of several other functional important molecules. We have demonstrated that memory T cells proliferate much more than naive cells when stimulated with CD3 mAb or pairs of CD2 mAb. Such enhanced responsiveness to receptor-mediated triggering is a novel mechanism for T cells which could facilitate memory cell response to specific antigen.
Furthermore, stimulation via either CD2 or CD3 results in substantial amounts of gamma interferon production by memory T cells but virtually none by naive cells; thus differentiation from naive to memory cells appears to be accompanied by a stable change in regulation of the gene for gamma interferon.
We are in the process of characterizing seven new monoclonal antibodies which are relevant to the foregoing phenotypic and functional studies and appear to identify at least two new molecules.

GPO 914-918

Project Description

Major Findings:

ICAM-1 was identified as a cell surface glycoprotein involved in LFA-1 dependent PMA-induced homotypic adhesion of lymphoblastoid B cell lines. We have been undertaking systematic analysis to determine if ICAM-1 is an LFA-1 ligand in antigen-independent T cell adhesion (AITA). We obtained not only the prototype monoclonal antibody (mAb) RR1/1 but also sought other potential αICAM-1 mAb based on fragmentary information on many other mAb. Our analysis of tissue distribution, functional activity and binding to purified ICAM-1 (see below) identified two additional ICAM-1-specific mAb: LB-2 (originally identified as a B cell activation antigen) and 84H10 (identified as a marker on myeloid leukemic cells). Observations from multiple laboratories regarding function, structure, tissue distribution, and genetics were unified by our finding that these three mAb detect a single molecule, namely ICAM-1. Furthermore, competitive binding assays indicate that these three mAb bind to two distinct sites on the ICAM-1 molecule. Antibody blocking studies indicate that mAb against both of these sites block the function of ICAM-1 in adhesion.

Although our previous functional studies (AR05067-12) indicated that ICAM-1 is a ligand for LFA-1, more direct analysis was undertaken using immunoaffinity purified ICAM-1. Studies of ICAM-1 immobilized on plastic surfaces demonstrated that the RR1/1, LB-1 and 84H10 mAb mentioned above all bound to ICAM-1, albeit to two sites on the molecule. LFA-1-expressing T cells (CTL), B cells (LCL) and myeloid cells (U937) all bind to immobilized ICAM-1. The binding is specifically inhibitable by αLFA-1 mAb and each of the αICAM-1 mAb. These studies demonstrate that ICAM-1 is a ligand for LFA-1-dependent cell adhesion. We analyzed the divalent cation requirements of LFA-1 dependent adhesion to purified ICAM-1 and directly compared those with the cation requirements of intercellular adhesion via the molecules LFA-1 and ICAM-1. The similarities between results in the two assay systems is striking: Mg^{++} alone is sufficient for optimal adhesion; Ca^{++} alone is not sufficient for adhesion but can synergize with suboptimal concentrations of Mg^{++}. The similarities between the two assay systems emphasize that the assay of cell binding to purified ICAM-1 faithfully reflects the biology of LFA-1 interactions which are involved in more complex intercellular interactions. Two divalent cation-binding sites may be involved: one which requires Mg^{++} and is saturated at concentrations below 0.1 mM and another which binds either Mg^{++} or Ca^{++} but with somewhat lower affinity than the first site. We are pursuing studies of LFA-1/ICAM-1 interaction in this model system of immobilized ICAM-1 since it reproduces not only the cation requirements but other important features observed in more complex cell interaction, such as the dissociation phase which occurs following the binding phase.

We have extended studies of human T cell subsets which differ in expression of adhesion molecules (AR05067-12), both with respect to phenotypic and functional analysis. Further analysis has confirmed our interpretation that these two subsets are human naive and memory cells. Human memory T cells are phenotypically distinguishable from naive T cells by increased expression

of six cell surface molecules (LFA-3, CD2, LFA-1, 4B4, UCHL1, and Pgp-1). Neonatal cord blood consists almost exclusively of naive cells expressing lower levels of these markers, which increase in expression following PHA-activation. Furthermore, adult cells of the "naive" phenotype also convert to the memory phenotype following PHA stimulation. As expected, only the high-expressing T cells in adults proliferate in response to recall antigens.

Now that human naive and memory cells can be distinguished phenotypically, functional properties of these important subsets have been investigated. We have demonstrated that memory T cells proliferate much more than naive cells when stimulated with CD3 mAb or pairs of CD2 mAb. Although the response to CD2 may be enhanced due to increased expression of CD2 on memory cells, the response to CD3 is not, since CD3 is expressed at equivalent levels on both subsets. Enhanced responsiveness to receptor-mediated triggering is a novel mechanism for T cells which could facilitate memory cell response to specific antigen. Furthermore, when triggered by several different agents, memory cells produce much more gamma interferon than do naive cells. The most dramatic differences are observed with stimulation via either CD2 or CD3 which results in substantial amounts of gamma interferon production by memory T cells but virtually none by naive cells. In response to PHA, the memory subset made greater than 5 fold more IFNγ than the naive cell subset, despite the fact that both subsets made equivalent amounts of IL2. This suggests that differentiation from naive to memory cells is accompanied by a stable change in regulation of the gene for gamma interferon.

We have begun to search for additional molecules involved in cell-cell adhesion. We have focused on two model systems: T cell binding to monocytes, (because it is apparent that this is a critical interaction in the immune response) and T cell rosetting with autologous erythrocytes (because it is a convenient model system in which additional adhesion molecules have been reported). Monoclonal antibodies (mAb) have been generated by immunizing mice with elutriated human monocytes, and seven mAb have been derived with the following useful properties: 1) three mAb which inhibit T cell rosetting with autologous erythrocytes; initial analysis indicates that they are directed against 2 distinct molecules, neither of which has previously been appreciated to be involved in autologous rosetting; 2) two mAb which inhibit T/monocyte adhesion, one of which is a molecule which is apparently undescribed, but critical to this adhesion; 3) one mAb which appears to identify a new molecule which is differentially regulated on human naive and memory T cells; 4) a marker of human granulocytes. Studies are in progress regarding further details of function of these molecules, their tissue distribution, and their structure.

Publications:

Makgoba MW, Shaw S, Gugel EA, Sanders ME. Human T cell rosetting is mediated by LFA-3 on autologous erythrocytes. J Immunol 1987;138:3587-3589.

Shaw S, Luce GG. The LFA-1 and CD2/LFA-3 pathways of antigen-independent human T cell adhesion. J Immunol 1987;139:1037-1045.

Makgoba MW, Sanders ME, Luce GE, Marlin SD, Springer TA, Clark EA, Mannoni PA, Shaw S. ICAM-1 a ligand for LFA-1-dependent adhesion of B, T and myeloid cells. Nature 1988;331:86-88.

Sanders ME, Makgoba MW, Luce GE, Shaw S. T cell adhesion receptors LFA-1 and CD2 and their ligands ICAM-1 and LFA-3: Analysis in adhesion, cell mediated lysis and as markers of T cell subsets. In: Kappler J, Davis M, eds. UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 73. New York: Alan R. Liss, Inc., 1988;269.

Sanders ME, Makgoba MW, Sussman EH, Luce GEG, Cossman J, Shaw S. Molecular pathways of adhesion in spontaneous rosetting of T lymphocytes to the Hodgkin's cell line L428. Cancer Res 1988;48:37-40.

Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, Shaw S. Three adhesion molecules (LFA-3, CD2, LFA-1) and three other molecules (UCHL1, 4B4, Pgp-1) show increased expression on a human T cell subset containing memory cells and characterized by enhanced gamma interferon production. J Immunol 1988;140:1401-1407.

Makgoba MW, Sanders ME, Luce GE, Gugel EA, Dustin ML, Springer TA, Shaw S. Functional evidence that intercellular adhesion molecule (ICAM-1) is a ligand for LFA-1 in cytotoxic T cell recognition. Eur J Immunol 1988;18:637-640.

Shaw S. Summary of session on HLA expression and adhesion molecules. ASHI Quarterly 1988;12:27.

Shaw, S. Immunology: A short course (book review). Immunol Today 1988;9:182.

Sanders ME, Makgoba MW, Sussman EH, Luce GE, Springer TA, Cossman J, Shaw S. Spontaneous rosetting of T lymphocytes to Reed-Sternberg cells is mediated by the CD2/LFA-3 and LFA-1/ICAM-1 pathways of antigen-independent adhesion. Ann NY Acad Sci 1987;in press.

Makgoba MW, Sanders ME, Luce GE, Gugel EA, Dustin ML, Springer TA, Shaw S. Intercellular adhesion molecule-1 (ICAM-1) monoclonal antibody inhibits cytotoxic T lymphocyte recognition. Ann NY Acad Sci 1987;in press.

Makgoba MW, Sanders ME, Luce GE, Dustin ML, Mannoni P, Clark EA, Springer TA, Shaw S. A cluster of antibodies (RR1/1, LB-2, and 84H10) which inhibit LFA-1-dependent lymphoid and myeloid cell adhesion bind intracellular adhesion molecule-1 (ICAM-1). In: Dupont B, ed. Immunobiology of HLA. Vol #2: Immunogenetics and Histocompatibility. New York: Springer-Verlag, 1988;in press.

Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, Shaw S. 1988. Coordinate enhanced expression of three adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (4B4, UCHL1, and Pgp-1) defines a human T cell subset containing memory cells and characterized by enhanced gamma interferon production. In: Dupont B, ed. Immunobiology of HLA. Vol #2: Immunogenetics and Histocompatibility. New York: Springer-Verlag, 1988;in press.

Sanders ME, Makgoba MW, Shaw S. Human naive and memory T cells: reinterpretation and further characterization of helper-inducer and suppressor-inducer subsets. Immunol Today 1988;in press.

Shaw S, Shimizu Y. The CD2/LFA-3 and LFA-1/ICAM-1 pathways of T cell interaction. Curr Opin Immunol 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09258-10 E

PERIOD COVERED
October 1, 1987 to September 30, 1988 formerly Z01 CB 05086-09 I

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	EIB, NCI
Others:	M. Vacchio	Bio. Lab. Tech.	EIB, NCI
	J. Berzofsky	Senior Investigator	MB, NCI

COOPERATING UNITS *(if any)*

Alison Finnegan, Rush-Presbyterian Saint Lukes, Chicago, IL

LAB/BRANCH
Experimental Immunology Branch

SECTION
Immunotherapy Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	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.)*

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 clones were restricted to recognizing NASE in the context of either $A_\alpha A_\beta$ or $E_\alpha E$ products. The antigen fine specificity of cloned NASE-specific T cells was also probed through the use of mutant NASE molecules and synthetic peptides corresponding to segments of NASE. A consistent correlation was found between the fine specificity of a given clone and its MHC restriction specificity. $A^b_\alpha A^b_\beta$ restricted clones were selectively responsive to peptide 91-110; $E^k_\alpha E^k_\beta$ restricted clones were responsive to peptide 81-100.

The nature of the immunogenic peptides which are processed and presented to T cells was further evaluated using a panel of variant NASE peptides. It was observed that negative (inhibitory) interactions appear to exist between amino acids in limited peptides which interfere with T cell responses. Single amino acid changes, by eliminating such apparent negative interactions, result in the restoration of T cell stimulatory ability in these peptides.

Project Description

Major Findings: Monoclonal T_H cell populations specific for NASE have been generated in BALB/c (H-2^d) and (B10xB10.A)F_1 (H-2^bxH-2^a) genotypes. Of the BALB/c clones tested, individual clones show MHC restriction for either I-A or I-E products. Individual (B10 x B10.A)F_1 clones respond to native NASE in association with either $A^b_\alpha A^b_\beta$ or $E^k_\alpha E^k_\beta$. When the fine specificity of these clones was analyzed employing NASE fragments and a series of overlapping 20 amino acid synthetic peptides corresponding to the NASE sequence, it was found that $A^b_\alpha A^b_\beta$ restricted clones were highly responsive to peptide 91-110 and not to other synthetic NASE peptide. In contrast, $E^k_\alpha E^k_\beta$ restricted clones were consistently responsive to peptide 81-100 and not to 91-110; certain of these $E^k_\alpha E^k_\beta$ restricted T cells expressed a cross reactivity with peptide 51-70. A BALB/c clone was responsive only to peptide 61-80. No other NASE peptides have revealed antigenic activity to date. Collectively, these findings demonstrate that NASE specific T cells are responsive to discrete peptides. Moreover, these findings suggest that in T cell recognition of a complex and highly foreign protein antigen, a limited number of peptide epitopes is preferentially recognized by T cells in association with a given Ia molecule.

More detailed analysis of T cell fine specificity in response to NASE peptides was carried out using variants in which a single amino acid was altered from its native or wild type form. This analysis revealed that clones with specificity for the same native peptide and self MHC determinant may nonetheless vary significantly in their fine specificity as assessed by responses to variant peptides. It was also observed that certain $E^k_\alpha E^k_\beta$ restricted clones responded to peptide 91-100 but not to 91-105. On the basis of this finding, it was suggested that interactions between amino acids in the 101-105 region with amino acids in the stimulatory 91-100 region were responsible for impaired ability to stimulate T cells. Consistent with this hypothesis, it was found that single amino acid changes including those in the 91-100 as well as those in the 101-105 region were capable of restoring stimulatory ability. Such changes appear to have their effect by eliminating inhibitory interactions among amino acid residues in the 91-105 peptide.

Proposed Course of Project: Cloned T cell populations and synthetic peptides will be employed to further analyze the mechanism for antigen fine specificity and MHC restriction. Variant peptides will be analyzed to more definitively identify antigenic sites on peptide antigens. Collaborative studies are in progress to identify any specific affinities in the binding of antigenic peptides with specific Ia molecules as a basis for selective antigen-Ia associations characteristic of Ir gene regulation.

The influence of non-MHC genes on anti-NASE responses will be studied. As noted in project Z01 CB 05106-07, expression of non-MHC Mls determinants can result in deletion of expression of certain V_β genes. The influence of non-MHC genes, including Mls^a and Mls^c, will therefore be studied in T cell responses to NASE. Both the peptide fine specificity of these responses and the patterns of T cell receptor V_α and V_β expression will be used to monitor the expressed repertoire by both functional and structural criteria.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09259-10 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 Formerly Z01 CB 05088-09 I

TITLE OF PROJECT *(80 characters 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	EIB, NCI
Others:	F. Hakim	Senior Staff Fellow	EIB, NCI
	C. S. Via	Medical Staff Fellow	EIB, NCI
	M. Fukuzawa	Visiting Fellow	EIB, NCI
	S. Sharrow	Chemist	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒x(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 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 may or may not induce GVHID, depending on the class I determinants required. Recognition of class II only abrogates $L3T4^+$ T helper cell responses but not $Lyt2^+$ T helper cell responses; recognition of class I and II results in loss of both $L3T4^+$ and $Lyt2^+$ T helper cell 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.

Inoculation of parental T cells into F_1 mice can also result in different immune abnormalities, depending on the donor and host strains used. Injection of C57BL/6 cells into $B6D2F_1$ mice resulted in extensive immune suppression, hypogammaglobulinema, and susceptibility to infection. Injection of DBA/2 cells into $B6D2F_1$ mice resulted in selective suppression of $L3T4^+$ T helper cell function, hypergammaglobulinema, and autoantibody production with SLE-like symptoms. The differences in these two forms of SLE were attributed to a defect in DBA/2 anti-F_1 CTL precursor frequency.

The parent-into-F_1 GVH reaction also results in severe defects in bone marrow stem cell function, as well as in a defect in the self MHC restriction ability of the F_1 thymus.

Project Description

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, and is associated with a selective loss of $CD4^+$ T helper cell function and of $CD4^+$ T cells. In contrast, class I + II-induced GVHID resulted in loss of self + X and allogeneic responses, and was associated with a loss of $CD4^+$ and $Lyt2^+$ T helper cell function. The induction of class II only GVHID required only $CD4^+$ parental cells, whereas induction of class I + II GVHID required $CD4^+$ and $CD8^+$ cells.

Protection against GVH-suppression could be induced by immunizing the F_1 mice to H-2 alloantigens prior to induction of suppression. This protection was antigen-specific. Such immunization prevented the loss of GVH-induced IL 2 receptors, and loss of IL 2 production. This protection could be demonstrated for a murine pathogen such as cytomegalovirus infection.

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 are lost whereas MHC allogeneic responses remain intact. Furthermore, the developmental stages of AIDS and class II GVHID result in a loss of $CD4^+$ T helper cells, respectively. It is also noteworthy that susceptibility to certain pathogens in both AIDS and in class II GVHID requires not only the loss of the "$T4^+$" helper cell population, but also "$T4^-$" T helper cells. This implies that the "$T4^-$" helpers are relevant for immune responses to antigens other than MHC alloantigens -- and to pathogenic agents in particular. Another parallel between AIDS development and class II GVHID is the polyclonal activation of B cells and autoantibody production. Thus, it may be that the very early immune signals that occur after HIV infection are similar to the immunostimulatory model of GVH, as well as those observed in autoimmune mouse strains.

We have also noted that certain class I + II GVHID situations result in functional defects that resemble class II GVHID only, i.e., in a selective loss of self + X, but retention of alloreactivity. These examples of GVHID appear to be auto-immune-like, and result in the production of anti-DNA antibodies and a lupus-like syndrome. The example that we have studied is DBA/2 into $B6D2F_1$ (compared with C57BL/6 into $B6D2F_1$). The B6 GVH model results in hypogammaglobulinema, profound immune deficiency that is not circumvented by IL 2 in vitro, suppressor cell activity that affects both $CD4^+$ and $CD8^+$ cell function, and susceptibility to immune cytomegalovirus infection. In contrast, the DBA/2 GVH model results in hypergammaglobulinema, anti-DNA antibodies, a deficiency and suppressor cell activity that is limited to $CD4^+$ T helper cell function and that is circumvented in vitro by IL 2, and resistance to cytomegalovirus infection. B10.D2 spleen cells into $B6D2F_1$ resembles the B6 GVH

model indicating the different forms of GVHID and not H-2 linked. Studies with (BxD) R. I. lines indicate that the difference is due to more than one non H-2-linked gene. The functional difference between DBA and B6 that we consider to be responsible for the two different forms of GVHID is that DBA/2 spleens contain 9-fold fewer anti-F_1 CTL precursors than to B6 spleens. Furthermore, the DBA GVH could be converted to the B6 GVH by increasing the inoculum size, and the B6 GVH could be converted to the DBA GVH by depletion of B6 $CD8^+$ cells. These findings suggest that this model of lupus is due to a deficiency in $CD8^+$ effector cells that would normally remove the source of antigenic stimulation and IL 2 production. Thus, a fundamental defect in SLE patients could be deficient effector function for the relevant antigen which leads to continuous and unopposed antigenic stimulation.

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, is not necessarily associated with GVH induced loss of CTL potential, and requires class II recognition only. Although class I recognition alone may 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.

Stem cell function in GVH mice is reduced by at least 10-fold, and the stem cell repopulation that does occur does not result in the development of functional T lymphocytes. The ability of the thymus of GVH mice to induce stem cells to mature into $CD4^+$ T helper cells is abrogated, but the ability of the thymus of the same GVH mice to induce differentiation of allospecific T helper cells remains intact. This defect appears to involve that component of the thymus that is responsible for MHC self-restriction and the discrimination between self and non-self.

Publications:

Iwasaki T, Hallam JA, Joseph LJ, Shearer GM. Antigen-specific protection against graft-vs-host induced immune deficiency. J. Immunol. 1987; 138:3604-10.

Via CS, Sharrow SO, Shearer GM. Role of cytotoxic T lymphocytes in the prevention of lupus-like disease occurring in a murine model of graft-vs-host disease. J. Immunol. 1987; 139:1840-49.

Moser M, Sharrow SO, Shearer GM. Role of $L3T4^+$ and $Lyt2^+$ donor cells in graft-versus-host immune deficiency induced across a class I, class II, or whole H-2 difference. J. Immunol. 1988; 140:2600-08.

Fukuzawa M, Shearer GM. Effect of graft-vs-host reaction on thymic function. Immunol. Res. 1988; in press.

Fukuzawa M, Shearer GM. Selective $L3T4^+$ T helper defect for the generation of cytotoxic effector cells in graft-vs-host, bone marrow reconstituted mice. N.Y. Acad. Sci., in press.

Fukuzawa M, Via CS, Shearer GM. Defective thymic education of L3T4$^+$ T helper cell function in graft-vs-host mice. J. Immunol. 1988; 141:in press.

Morse HC, Yetter RA, Shearer GM. Murine models of immunosuppression in relation to AIDS. In: Gallo RC, ed. HTLV I, II, and III, and their associated human diseases. Boston: Martinus Nijhoff Publishing, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09260-08 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 Formerly Z01 CB 05099-07 I

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	EIB, NCI
Others:	D. M. Segal	Senior Investigator	EIB, NCI
	J. Titus	Chemist	EIB, NCI
	C. Via	Medical Staff Fellow	EIB, NCI
	S. Sharrow	Chemist	EIB, NCI

COOPERATING UNITS *(if any)*

J. D. Shanley, V.A. Hospital, Newington, CT

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Injection of F_1 hybrid mice with either murine cytomegalovirus (MCMV) or parental spleen cells (graft-versus-host reaction - GVHR) results 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. In contrast, there was no detectable synergy between class II GVH and MCMV. 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. The combination of MCMV and GVHR also resulted in interstitial pneumonitis, whereas either insult alone had no detectable pathogenic effect on the lungs. These studies permit the investigation of the immunosuppression of MCMV infection and the possibility consequences of CMV infection coupled with a GVHR. The anti-viral drug, DHPG, inhibited viral replication but did not prevent MCMV from synergizing with GVH.

Lethally irradiated mice grafted with syngeneic bone marrow develop tumors between 18 and 24 months after irradiation. Mice infected with MCMV two weeks after irradiation did not develop tumors.

Project Description

Major Findings: To investigate possible synergistic effects between MCMV and GVH, different doses of parental spleen cells were injected into adult, immune-intact F_1 mice (differing from the donor at MHC class I + II) with or without MCMV infection. Splenomegaly and immunodeficiency were observed in mice exposed to both insults, even in mice injected with very low doses of parental cells. Mice infected with MCMV and injected with parental cells died of interstitial pneumonia 16-20 days after injection, whereas mice exposed to either insult alone appeared to be healthy. The interstitial pneumonitis was not related to levels of viral replication in the lungs, and may have been the result of more intensive pulmonary GVH activity. To investigate this phenomenon further, MCMV/GVH pneumonitis was initiated in F_1 mice as outlined above, and the number, origin, and phenotype of the cells recovered from bronchoalveolar lavage was determined during pneumonitis. Our findings indicate an approximate 10-fold increase in cellularity in lungs of the MCMV-GVH mice compared to mice exposed to either insult alone. More than 80% of these cells were T lymphocytes of donor origin, of which approximately half were $L3T4^+$ and half $Lyt2^+$. Attempts to determine the antigenic specificity of these donor-derived T cells have not yet been successful.

Because of the synergistic effects of MCMV/GVH on pneumonitis and survival, experiments were performed to determine whether exposure to MCMV prior to the induction of a GVHR would have an effect on surviva. F_1 mice were injected at 3 weekly intervals with live MCMV and then injected with parental spleen cells and challenged with MCMV 1 week later. All mice treated in this way survived. MCMV-specific antibody titers developed during the priming phase, but decreased after GVH induction. Ninety per cent of the mice that were not primed to MCMV, died after GVH and CMV challenge, and no anti-CMV titers were detected. Interestingly, 44% of the mice primed to CMV and injected with parental cell, but which were not given a post-GVH challenge with MCMV died. These findings suggest that immunization to CMV prior to a situation in which GVH is likely to occur could have beneficial effects in preventing interstitial pneumonia.

To determine whether the MCMV-GVH synergy was more pronounced when MCMV was induced by donor leukocytes or was resident in the host at the time of GVH induction, prospective donors of parental spleen cells were infected with MCMV at various times prior to injection into F_1 mice, and alternatively, prospective F_1 hosts of GVHR were infected with MCMV at different times prior to injection of parental cells. The results indicate that MCMV infection not only reduces the number of parental spleen cells required to induce graft-versus-host immune deficiency (GVHID) but accelerates the onset of GVHID, which occurs as early as 3 days after cell and virus challenge. To determine whether MCMV infection exerts this synergistic effect primarily through the donor or the host component, we examined the effect of MCMV infection of either donor mice or recipient mice at 3, 10 and 17 days prior to spleen cell transfer. When donor mice were infected with MCMV three days prior to cell transfer, the ability of donor cells to induce GVHID was reduced. In contrast, MCMV infection of the recipients three days prior to cell transfer increased their susceptibility to GVHID induction. Infection of either donor or host

mice 10 days or 17 days prior to parental spleen cell transfer has little effect on the ability to either induce or resist GVHID when compared to sham infected mice. Thus, acute MCMV infection can modulate the severity of GVHID, depending on whether it is the donor or the host that is infected. The ability of acute MCMV to alter the course and severity of GVHID may be relevant for human bone marrow transplants in which preceding CMV infection has been associated with chronic GVH. In this setting, CMV may lower the threshold necessary to induce a GVH reaction.

Our observations that: a) MCMV infection is often associated with enhanced T effector cell immunity to non-CMV antigens; and b) MCMV can synergize with low doses of donor leukocytes to induce GVH disease raises the possibility that MCMV infection can initiate a T helper signal with the production of lymphokines. Such a helper signal must be generated by MCMV-specific, MHC self-restricted helpers with the production of IL-2 (and other T helper factors), which could enhance the CD8 component of T cell immunity. As noted in Section I, a class I + II GVHR results in the loss of both CD4 and CD8 T function, whereas a class II GVH results in a selective loss of CD4 T helper function. A class I GVH that is limited to recognition of H-2D^{b} or H-2D^{d} results in no detectable GVH, as determined by host T cell function. We have used the differential effects of these three types of GVH on T cell immune function to determine whether CMV infection is providing a "class I" or "class II" type of signal in its synergistic action with GVH. We found that MCMV infection had no additional effect on a class II GVH, i.e., with or without CMV the class II GVH abrogated only CD4 function. In contrast, when MCMV was given in conjunction with the H-2D GVH (which had no detectable effect alone), the combination of MCMV and H-2D class I recognition abrogated both CD4 and CD8 T cell function - characteristic of a class I + II GVH effect. Studies of this type, as well as experiments which demonstrate the helper-like effects of MCMV recognition by T cells in vitro may elucidate the immune mechanisms responsible for the augmenting effects of MCMV in synergy with GVH, and in other models of immune enhancement.

In some experiments we have attempted to design protocols in which lethally irradiated mice were transplanted with allogeneic bone marrow with or without T cells and with or without CMV infection. Such experimental designs would more closely approximate the situation encountered in human marrow transplantation. Due to the high incidence of mortality in CMV-infected, GVH, irradiated mice, these complex experiments have not been successful. However, the mice that survived long-term were those that were irradiated and grafted with syngeneic bone marrow, with or without CMV infection. Between 12 and 18 months after irradiation and syngeneic marrow grafting, these mice began to develop sarcomas, probably as a result of exposure to 900R of whole-body irradiation. However, the development of the sarcomas (5/11) appeared to be limited to those mice that received 900R, and syngeneic marrow, but not MCMV. Mice that were exposed to MCMV, in addition to receiving irradiation and syngeneic marrow did not develop detectable tumors (0/17). Although these results (compilation of two experiments) are incomplete and need to be redesigned and repeated, the preliminary observations raise the possibility that exposure to MCMV has provided some element of immune protection against the development of the sarcomas. One possibility is that the mechanism responsible

for enhanced, immune reactivity of MCMV-infected mice noted above could contribute to the prevention or delayed development of these radiation-induced tumors.

We have also used the MCMV model to test the susceptibility of mice whose CD4 T helper pathway was either suppressed (as in the DBA GVH model described in Section I), or failed to develop function (as in GVH-BMT mice described in Section IV). The results indicate that despite our inability to demonstrate CD4 T helper cell function in vitro (by IL-2 production and CD4-dependent CTL generation), CD4-deficient mice did not die from MCMV infection, whereas mice deficient in both CD4 and CD8 function died within 3 weeks of MCMV infection. It is noteworthy that the CD4-deficient mice did not clear virus nor produce anti-CMV antibodies; nevertheless they did not die. These findings raise the possibility that immune mechanisms other than CD4 T helper cells and anti-CMV antibodies provide some protection to MCMV infected mice.

Publications:

Shanley JD, Via CS, Sharrow SO, Shearer GM. Intersitial pneumonitis during murine cytomegolovirus infection and graft-vs-host reaction: characterization of bronchoalveolar lavage cells. Transplantation. 1987; 44:658-62.

Via CS, Shanley JD, Lang P, Shearer GM. Altered threshold for the induction of graft-versus-host immunodeficiency following murine cytomegalovirus infection: host and donor contributions. Transplantation. 1988; in press.

Via CS, Shanley JD, Weatherly BR, Lang P, Shearer GM. Altered threshold for the induction of graft-versus-host immunodeficiency following murine cytomegalovirus infection: Host and donor contributions. Transplantation. 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09263-07 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05103-06 I

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

Structure and Function of Cytotoxic and Helper 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	EIB, NCI
Others:	K. Andrews	Microbiologist	EIB, NCI
	W. Munger	Guest Researcher	EIB, NCI
	R. Hodes	Senior Investigator	EIB, NCI
	M. Taplits	Medical Staff Fellow	EIB, NCI
	R. Gress	Senior Investigator	EIB, NCI
	M. Hayes	IRTA Fellow	EIB, NCI
	M. Kolber	Medical Staff Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	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.)*

We have found that granule protease granzyme A can be inactivated by pretreatment of intact CTL with the covalent inhibitor PMSF. Because this reagent reacts with the active site serine in a pH dependent reaction, efficient inactivation also requires the presence of agents which raise the pH of acidic intracellular compartments. Thus these secretory granules have a low pH interior, as has been found in most other cells. Interestingly, inactivation of granzyme A in intact CTL does not significantly inhibit the lytic efficiency of the CTL, implying that this protease activity may not be required for cytotoxicity. However, target DNA breakdown is greatly reduced when the CTL has been pretreated with PMSF to inactivate granule proteases. We have found that cytotoxicity mediated by purified granules from CTL or NK tumors is accompanied by target DNA breakdown, and that this appears to require both cytolysin and another granule component. We have studied this other granule component(s) by assaying DNA release from detergent-isolated nuclei. In CTL granules this activity is dramatically inhibited by low concentrations of PMSF and DFP, which are specific inhibitors of serine proteases. This reinforces the above results suggesting a role for serine proteases in target cell DNA breakdown. We have developed new quantitative methods for measurement of secretion from lymphocytes using fluorescent dyes such as quinacrine which localize in the acidic interior of the granules.

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

Project Description

Major findings:

We have found that granule protease granzyme A can be inactivated by pretreatment of intact CTL with the covalent inhibitor PMSF. Because this reagent reacts with the active site serine in a pH dependent reaction, efficient inactivation also requires the presence of agents which raise the pH of acidic intracellular compartments. Thus these secretory granules have a low pH interior, as has been found in most other cells. Interestingly, inactivation of granzyme A in intact CTL does not significantly inhibit the lytic efficiency of the CTL, implying that this protease activity may not be required for cytotoxicity. However, target DNA breakdown is greatly reduced when the CTL has been pretreated with PMSF to inactivate granule proteases. This suggests that a granule protease may be involved in target DNA breakdown. In another approach to this issue, we have considered whether granule components mediate target DNA breakdown in the presence of detergent, hypothesizing that normally such granule moieties could gain access to the target cell via the cytolysin induced pores. We have found that cytotoxicity mediated by purified granules from CTL or NK tumors is accompanied by target DNA breakdown, and that this appears to require both cytolysin and another granule component. We have studied this other granule component(s) by assaying DNA release from detergent-isolated nuclei. In NK tumor granules this activity is found in two components, which are minor protein components distinct from cytolysin and granzyme A. In CTL granules this activity is dramatically inhibited by low concentrations of PMSF and DFP, which are specific inhibitors of serine proteases. This reinforces the above results suggesting a role for serine proteases in target cell DNA breakdown.

We have developed a model system to explore the possibility that lymphocyte granule exocytosis occurs into a synapse-like zone created between the lymphocyte and an attached triggering cell. When allowed to settle onto glass surfaces coated with anti-T3 antibody, cloned mouse CTL and T helper cells exclude the penetration of antibodies from a region of substrate underneath themselves. When these cells are attached to the substrate via some other antibodies, such zones are not detectable by our fluorescence technique. Similarly, splenic T cells or thymocytes attached via anti-T3 antibodies very rarely show a zone of exclusion, suggesting that this ability may result from a differentiation of the T cells. The formation of these synapse-like junctions is not blocked by drugs which clock energy production or interfere with cytoskeletal protein function. At present we speculate that junctional regions where diffusion from the medium is limited may form by the creation of two-dimensional antigen-antibody complex meshes. These may be important for polarized secretion of lymphocyte mediators.

In collaboration with Dr. Ruth Angeletti, we have used anti-peptide antibodies made against two different regions of chromogranin A to identify several forms of this protein in the granules of rat LGL tumor cells by doing Western blots on Percoll gradient fractions. Using the Western blot technique with whole cell detergent lysates, we have detected chromogranin A in cloned CTL lines, cloned helper T cell lines, rat NK tumor lines, and purified rat blood LGL cells. Rat blood T lymphocytes were negative. Thus we find that lymphocytes which have well developed secretory granules appear to express

chromogranin A. These results show that the expression of this protein is not unique to the neuroendrocrine system, and they reinforce other lines of evidence that immune and nervous systems are interlinked.

We have sought to develop more general methods for studying the regulated secretion event in lymphoid cells. In one approach, we have utilized weak base fluorescent dyes such as quinacrine which localize in the acidic interior of the granules. We showed that secretion triggered by ionomycin or the IgE receptor could be followed by measuring changes in the uptake of quinacrine from the medium into RBL cells, as predicted from a theoretical model of dye partitioning. This principle has also been utilized with flow microfluorimetry, which has allowed us to detect degranulation in human CTL after contact with anti-T3 immobilized on polystyrene beads.

Proposed course:

The nuclear DNA releasing activity in the granules of CTL and NK tumor cells will be extensively purified by standard biochemical techniques, using the assay of release of ^{125}I-DNA from nuclei in the presence of Triton X100. Since this activity in CTL granules is extensively inhibited by low concentrations of DFP and PMSF, we will also use labelled DFP to identify the functionally relevant component. Other serine protease inhibitors which block the nuclear DNA releasing activity should block DFP labeling of the relevant component with similar titration curves.

The simplest explanation of target DNA breakdown by the granule exocytosis model is that granule components enter the nucleus and directly degrade DNA. In support of this possibility, we can measure a granule DNAse activity at neutral pH. By this model, a granule protease could provide enhanced DNA accessibility to the DNAse. In order to test the plausibility of granule DNAse acting in this way, we plan to use TNP modified resealed red cell ghosts as targets for CTL using the anti-T3xanti-DNP heteroconjugate antibody prepared by Dr. David Segal. We will reseal supercoiled plasmid pUC DNA inside the ghosts, and see if the DNA becomes nicked or degraded when the ghosts are attacked by CTL. (We have already shown that CTL effectively kill TNP-RBC with this heteroconjugate in a calcium dependent reaction.)

In collaboration with Dr. Hodes' laboratory, we plan to make a more concerted effort to characterize the molecules present in T helper lymphocyte granules by making larger granule preparations and doing both biochemical analysis and assaying for lymphokines. Biochemical analysis is difficult because of the small amounts of purified lymphocyte granules available from most clones. The helper clones used secrete IL-4, but it is not known whether this lymphokine (or any other lymphokine) is secreted by the regulated or constitutive secretory pathway (although evidence for a polarized secretory pathway for IL-4 was recently published.

We plan to carry out much more extensive investigation of the phenomenon of "synapse" formation by lymphocytes. Using the same basic technique, we are investigating antibodies against a range of cell surface antigens to ascertain

which will trigger the formation of an excluded zone along the adhering surface. We will concentrate on antibodies which are known to activate T cells (such as Ly 6 and Thy 1) but also look for other control antibodies which act like the anti-class 1 MHC antibodies. We will also look at human lymphocytes, both LGL and T cell lines. We will test a selection of the anti-human lymphocyte monoclonal abs. A preliminary look showed that human LGL did form an excluded zone with glass coated with IgG, as might be expected for this model ADCC surface. We will try to get some feeling for the size of molecules which are excluded from this region by using labeled protein G (MW 34 kd) instead of an IgG antibody (MW 150 kd) as a probe of the area under the cell. We will also examine various metabolic inhibitors and other drugs to ascertain what cellular processes are involved in the formation of such excluded zones. Finally we will attempt to demonstrate whether granule exocytosis occurs into this region by coating the surface with a substrate for granzyme A and seeing if it becomes selectively hydrolyzed on this patch of surface.

Publications:

Henkart P, Henkart M, Hodes R, Taplits M. Secretory processes in lymphocyte function. Bioscience Reports 1987;7:345-353.

Henkart PA, Berrebi GA, Takayama H, Munger WE, Sitkovsky MV. Biochemical and functional properties of serine esterases in acidic cytoplasmic granules of cytotoxic T lymphocytes. J Immunol 1987;139:2398-2405.

Leo O, Foo M, Henkart PA, Perez P, Shinohara N, Segal DM, Bluestone JA. Role of accessory molecules in signal transduction of cytolytic T lymphocytes by anti-T cell receptor and anti-Ly-6.2C monoclonal antibodies. J Immunol 1987; 139:3556-3563.

Reynolds CW, Reichardt D, Henkart M, Millard P, Henkart P. Inhibition of NK and ADCC activity by antibodies against purified cytoplasmic granules from rat LGL tumors. J Leukocyte Biol 1987;42:642-652.

Kolber MA, Henkart PA. Quantitation of secretion by rat basophilic leukemic cells by measurements of quinacrine uptake. Biochim Biophys Acta 1988;939: 459-466.

Colamonici OR, Ang S, Quinones R, Henkart P, Heikkila R, Gress R, Felix C, Kirsch I, Longo D, Marti G, Seidman JG, Neckers LM. IL-2 dependent expansion of CD3+ large granular lymphocytes expressing T cell receptor gamma-delta: Evidence for a functional receptor by anti-CD3 activation of cytolysis. J Immunol 1988;140:2527-2533.

Munger WE, Berrebi GA, Henkart, PA. Possible involvement of CTL granule proteases in target cell DNA breakdown. Immunol Rev 1988;103, in press.

Taplits MS, Henkart PA, Hodes, RJ. T helper cell cytoplasmic granules: Exocytosis in response to activation via the T cell receptor. J Immunol 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09264-01 E

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Effects of Cyclosporin A on T Lymphocyte Regulation and Development

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

PI:	G. M. Shearer	Senior Investigator	EIB, NCI
Others:	M. Fukuzawa	Visiting Fellow	EIB, NCI
	A. Kosugi	Visiting Fellow	EIB, NCI
	M. Clerici	Visiting Fellow	EIB, NCI
	A. Palestine	Senior Investigator	CB, NEI

COOPERATING UNITS *(if any)*

P. Kimmel, Renal Unit, George Washington Univ. School of Medicine

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B,A

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

As an extension of our GVH work, my laboratory has recently begun to investigate the effects of CSA on peripheral T cell immune function, as well as on the ability of CSA-treated mice to develop functional T cells from bone marrow stem cells. Multiple injections of different doses of CSA (30 or 75 mg/kg) had different effects on peripheral T cell function. The lower dose of CSA selectively abrogated CD4 T helper cell function, but not CD8 T helper or effector function. Suppressor cells were detected that suppressed the CD4 but not CD8 T cell function of normal mice. Seventy-five mg/kg abolished all T cell function tested, and suppressor cells were detected which had a similar effect on T cells from normal mice. Mice treated with these doses of CSA were unable to develop CD4 T helper cell function from normal stem cells, and this defect was due to CSA-induced thymic damage. These results indicate that abrogation of T cell function by CSA can be selective, depending on the dose of CSA administered. This study also suggests that in vitro CD4 and CD8 T cell functional analysis should be performed in human allograft recipients undergoing CSA treatment, and raises the possibility that not all human T cell functional pathways will be abrogated by a particular CSA protocol in allograft patients. Recent in vitro data suggest that distinct functional T cell subsets of human PBL will be differentially susceptible to CSA.

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

Project Description

Major Findings: Mice exposed to different daily doses of CSA of 30 or 75 mg/kg/day were tested for in vitro generated splenic T cell responses to antigens that require CD4 T helpers (TNP-self and CD4-mediated ALLO) and to those that can use $CD8^+$ T helpers (ALLO). Functional tests performed one day after termination of CSA indicated that 30 mg/kg CSA selectively abolished CD4 T helper function whereas 75 mg/kg CSA abrogated all T helper and effector function. Immune function returned within 2 weeks of termination of CSA. At the 30 mg/kg dose, radiosensitive suppressor T cells of the Lyt2 phenotype were detected which selectively suppressed $CD4^+$ T helper function of normal syngeneic spleen cells. At 75 mg/kg CSA, radioresistant, non-T suppressor cell activity was detected that suppressed both CD4 and CD8 function of normal spleen cells. That this "suppressor phenomenon" induced by the 30 mg/kg dose was due to cells with suppressor activity and was not due to the trivial explanation of "CSA carryover" was demonstrated by the fact that suppressor activity was radiosensitive, and abrogated by anti-Thy 1.2 or anti-Lyt2 antibodies and complement. These findings indicate that different subsets of T cells can be differentially affected by CSA, depending on the dose used which could account for the failure of some studies to detect in vitro functional effects of CSA. Knowledge of our findings could be important in designing protocols for allograft patients. Our observations that the immune deficiency induced by CSA is associated with suppressor cell activation should be important in elucidating the mechanism(s) responsible for the CSA effect on preventing graft rejection.

In vitro studies in which different doses of CSA were added to primary sensitization cultures of murine spleen cells and human PBL indicate a similar pattern. Thus, lower doses of CSA selectively abrogated CD4-mediated helper function, whereas higher doses of CSA abolished all helper function. These results suggest that a similar CSA dose-dependent selective effect on $CD4^+$ T helper function exists for human T cells.

The GVH effects that we observed on thymic potential to develop $CD4^+$ T helper cell function, and the effects of CSA reported on T cell development in irradiated marrow-reconstituted animals suggested that CSA treated mice that are subsequently irradiated and marrow grafted (CSA-BMT) would fail to develop $CD4^+$ T helper cell function. This prediction was confirmed, as CSA-BMT mice exhibited normal $CD8^+$ T helper and effector function, but failed to show CD4 T helper function. Similar to the GVH-BMT mice described above, the phenomenon: a) was not associated with suppressor cells; b) was not due to a defect in APC function; c) was not associated with a reduction in $L3T4^+$ or $Lyt2^+$ cells nor T3 expression; d) was circumvented in vitro by T cell helper factors or $L3T4^+$ cells from untreated mice; and e) was corrected in vivo by a neonatal thymus graft. These findings of the effects of CSA on thymic function complement studies of others, indicate that CSA can have a profound effect on the thymus, and provide another model (similar to that described in Section IV) for investigating the role of the thymus on T cell functional development.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09265-07 E

PERIOD COVERED
October 1, 1987 to September 30, 1988 formerly Z01 CB 05106-06 I

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

PI:	R. J. Hodes	Chief, Immunotherapy Section	EIB, NCI
Others:	R. Abe	Visiting Fellow	EIB, NCI
	M. Vacchio	Bio. Lab. Tech.	EIB, NCI

COOPERATING UNITS *(if any)*
Food and Drug Administration

LAB/BRANCH
Experimental Immunology Branch

SECTION
Immunotherapy Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

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 nature of polymorphism and allelism were re-evaluated in the minor lymphocyte stimulating (Mls) system. Mls determinants are defined by the ability to stimulate primary proliferative T cell responses between major histocompatibility complex (MHC) identical cells. Originally, Mls was described as a single locus system involving at least four polymorphic alleles. Proliferating T cell clones were generated which were specific for Mls^a, Mls^c, or Mls^d. These, in combination with primary proliferative T cell responses, were then employed to analyze the relationship between Mls^a, Mls^c, and Mls^d determinants. It was found that Mls^d cells appear to express the sum of Mls^a and Mls^c determinants. In addition, formal genetic analyses were carried out to identify the relationship between the genes encoding Mls^a, Mls^c, and Mls^d. It was found that Mls^a and Mls^c are encoded by non-allelic and in fact unlinked genes. Moreover, an Mls^d strain expresses independently the products of unlinked Mls^a-like and Mls^c-like genes. Thus, in contrast to previous understanding, the Mls system is composed of the products of at least two unlinked loci, with no evidence for structural polymorphism at either locus at the present time.

An analysis of T cell receptor (TCR) expression in Mls^c reactive T cells demonstrated a striking association of this reactivity with expression of the Vβ3 gene product. This was true both for T cell clones generated by repeated stimulation with Mls^c stimulators as well as for antigen specific T cells with coincidental reactivity to Mls^c. In addition, it was demonstrated that mouse strains which express Mls^c delete expression of Vβ3 in their mature peripheral T cell populations. This failure to express Vβ3 presumably reflects a consequence of the maintenance of tolerance to self Mls^c products. These findings provide a basis for the understanding of high T cell precursor frequency for Mls^c products, reflecting the overall expression of Vβ3 in the T cell repertoire. Moreover, they demonstrate the importance of these products in selection of the antigen-specific T cell repertoire.

Project Description

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. Series of T cell clones were generated which are specific for Mls^a, Mls^c, or Mls^d. The Mls^a and Mls^c specificities of these clones were confirmed by use of panels of inbred strains, recombinant inbred strains, and formal segregation analysis in which the responses of clones were compared with the primary (definitive) T cell responses to MHC identical cells. This analysis demonstrated that Mls^a and Mls^c were non-crossreactive as defined by panels of T cell clones, and therefore that the Mls system as defined is indeed polymorphic. In addition, it was demonstrated that Mls^d cells express both Mls^a and Mls^c determinants.

2) These studies suggested the possibility that Mls^d might in fact represent the co-expression of Mls^a and Mls^c determinants, a possibility which would imply that Mls^a and Mls^c were encoded by non-allelic genes. In order to test this possibility, a formal segregation analysis was carried out in which (Mls^a x Mls^c)F_1 x Mls^b progeny were typed in primary MLR as well as in responses of Mls^a specific and Mls^c specific T cell clones. The results of these studies demonstrated conclusively that the genes encoding Mls^a and Mls^c determinants are non-allelic and in fact are unlinked. Additional analysis of the progeny from a (Mls^d x Mls^b)F_1 x Mls^b breeding indicated that Mls^d strains express the products of unlinked genes which are respectively Mls^a-like and Mls^c-like. Thus the original concept of Mls as a single locus, multi-allelic polymorphic system is not sufficient to explain these findings. A reappraisal of these results suggests the novel concept that Mls is in fact composed of the products of at least two non-allelic and unlinked genes, with no definitive evidence at present for polymorphism at either of these loci.

3) The influence of MHC gene products in the recognition of Mls was evaluated. It was found that Mls^c is highly stimulatory to T cells when it is presented on stimulatory cells which also express $H\text{-}2^k$ but not $H\text{-}2^b$ gene products. Experiments employing intra-H-2 recombinant strains demonstrated the importance of I-E region gene products in this phenomenon. Studies employing monoclonal anti-I-A and I-E antibodies also confirmed the importance of these class II molecules in recognition of Mls^c determinants. A consequence of this MHC influence on Mls stimulatory ability was the re-typing of Mls genotype in a number of strains. The expression of Mls^c determinants was not previously identified in these strains due to their failure to express a permissive MHC type.

4) The expression of T cell receptor (TCR) V_α and V_β chains was assessed in Mls^c-specific T cell clones. These included both clones which were generated by repeated stimulation with Mls^c determinants and clones specific for the antigen pigeon cytochrome c which were crossreactive with Mls^c. $V_\beta 3$ was expressed in 12 of the 13 Mls^c specific clones analyzed. Although $V_\beta 11$ usage was predominant in the cytochrome c reactive T cells, it was not expressed in the directly generated Mls^c specific T cells. The striking association suggests that expression of $V_\beta 3$ products alone, independent of other α or β chain polymorphisms, is sufficient to determine T cell specificity for Mls^c. It was also observed that strains which themselves express Mls^c, whether homozygous or heterozygous, delete the expression of $V_\beta 3$ from their peripheral T cell repertoires. This finding is consistent with the interpretation that maintenance of self tolerance to Mls^c determinants leads to deletion of $V_\beta 3$ expressing T cells.

Proposed Course of Research: Antibodies specific for T cell receptor structures will be utilized to determine whether the receptors used in recognition of Mls^a and Mls^c are the same or different from those used in recognition of other specificities. Attempts will be made to establish monoclonal cell lines expressing Mls^a determinants and to identify Mls-congenic strains of mice. These resources together will be employed in an attempt to generate monoclonal antibodies specific for Mls products and ultimately to characterize the Mls structures involved in T cell activation. 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. The observed deletion of $V_\beta 3$ expressing T cells from Mls^c animals will be employed as a system in which to study the process of self-non-self discrimination. The deletion of $V_\beta 3$ expressing T cells will be studied in thymic and bone marrow chimeras.

Publications:

Bluestone JA, Pescovitz MD, Frels WI, Singer DS, Hodes RJ. Cytotoxic T lymphocyte recognition of xenogeneic MHC antigen expressed in transgenic mice. Eur J Immunol 1987;17:1035-1042.

Abe R, Ryan JJ, Hodes RJ. Mls is not a single gene, allelic system: Mls^a and Mls^c are the products of non-allelic, unlinked genes. J Exp Med 1987; 166:1150-1155.

Abe R, Hodes RJ. Genetic analysis of serologically undefined determinants: A T cell "clonological" analysis of the Mls system. Currents Topics of Microbiology and Immunology 1987; in press.

Abe R, Hodes RJ. A reappraisal of Mls genetics. J Immunogenetics 1988; in press.

Abe R, Hodes RJ. Mls determinants recognized by T cells. J Immunogenetics 1988; in press.

Seldin MF, Abe R, Steinberg AD, Hodes RJ, Morse HC. Genetic relationships of Mls^a among polymorphic loci on distal mouse chromosome 1. J Immunogenetics 1988; in press.

Abe R, Hodes RJ. T cell recognition of Mls^c. I. Influence of MHC gene products in Mls^c-specific T cell recognition. J Immunol 1988; in press.

Abe R, Hodes RJ. The Mls system. Non-MHC genes which encode strong T cell stimulatory determinants. Immunology Today 1988; in press.

Abe R, Hodes RJ. The expression of Mls^c determinants on Mls^a, Mls^b, and Mls^x prototypic strains. Immunogenetics 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09266-06 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05108-05 I

TITLE OF PROJECT *(80 characters 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	EIB, NCI
Others:	M. Taplitz	Medical Staff Fellow	EIB, NCI
	K. Hathcock	Chemist	EIB, NCI
	D. Segal	Senior Investigator	EIB, NCI
	R. Guy	Visiting Fellow	EIB, NCI
	P. Henkart	Senior Investigator	EIB, NCI

COOPERATING UNITS *(if any)*

Department of Immunology, University of Tokyo, Tokyo, Japan

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	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.)*

B cell antibody responses can be activated by helper T cells through two distinct pathways. Individual cloned T helper cell populations are in fact capable of mediating both MHC-restricted and non-MHC-restricted pathways of B cell activation. It was subsequently demonstrated that cloned lines of Lyt 1^+ 2^- $L3T4^+$ antigen specific and MHC restricted suppressor cells can mediate suppressor effector function in these T dependent antibody responses.

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) and the CRI_A dominant response to Ars. It was found that cloned populations of carrier specific and MHC restricted T helper cells were capable of supporting T15 or CRI_A idiotype dominant responses in 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.

A role of both specific and non specific T cell derived signals was demonstrated for activation of B cell antibody responses. It was shown that the supernatants of activated cloned T helper cells were capable of replacing these T cells in the antigen specific and MHC restricted induction of B cell IgG antibody responses. Two distinct and synergizing activities were identified in this supernatant: Interleukin 4, and an antigen-specific and MHC-restricted soluble factor. It was demonstrated that this latter factor can be affinity purified employing monoclonal antibodies specific for T cell receptor Vβ8 determinants. A mechanism for the rapid and directed secretion of mediators by T help was also demonstrated by analysis of granule exocytosis by T helper cells. Cloned helper cells, in response to T cell receptor mediated stimuli but not in response to IL2, rapidly secrete the contents of cytoplasmic granules. This provides a potential mechanism for the transmission of B cell activating stimuli from helper T cells to target B cells.

GPO 914-918

Project Description

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: one mediated by Lyt 1^+2^- T_S cells which are capable of suppressing responses in an antigen non-specific manner, and one mediated by Lyt 1^-2^+ T suppressor cells which function in an antigen-specific effector pathway.

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.

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. Similarly, both heterogeneous and cloned carrier specific T helper populations were capable of eliciting Ars specific antibody responses from hapten-primed B cells in response to the antigen Ars-KLH. In this situation as well, cloned helper T cells were sufficient to generate optimally CRI_A idiotype dominant responses.

5. Mechanisms of Cognate T Helper Function.

The nature of the signals transmitted by T helper cells to responding B cells in the course of MHC restricted and antigen specific pathways of B cell activation was studied. Cloned helper T cells were activated in vitro either through exposure to specific antigen and antigen presenting cells or through stimulation with solid phase immobilized antibody to the T cell receptor T3 complex. The cell free supernatant generated under these conditions was capable of functioning in an antigen specific and MHC restricted fashion to activate B cell IgG antibody responses. The helper activity of these supernatants was inhibited by monoclonal antibodies specific for IL4, demonstrating that this lymphokine is one of the essential and synergizing components of the helper cell supernatant. A second component, with the properties of both antigen specificity and MHC restriction, was affinity purified using monoclonal antibodies specific for the $V\beta 8$ determinant of the T cell receptor. These findings demonstrate that both specific and non specific T cell products are capable of synergizing in the activation of B cells. Moreover, they strongly suggest that a cell-free form of the T cell receptor is capable of binding to its specific ligand and mediating T helper cell function.

In a separate but related set of studies, it was demonstrated that cloned T helper cells bear cytoplasmic granules which contain a series of detectable enzymatic activities. When stimulated with specific antigen or with antibodies directed at T cell receptor structures, but not when activated with IL2, these cloned T helper cells undergo a rapid granule exocytosis. This secretory process provides a mechanism whereby prestored granules containing potentially relevant biologic mediators may play a role in the T helper cell function.

Proposed Course of Project: The signals involved in T cell-mediated activation of B cell responses will be most intensively studied through the characterization of cell free T helper products. The antigen specific T helper factor will be assessed by batch affinity purification. Attempts will then be made to biochemically characterize this product and in particular to establish its relationship to the cell surface T cell receptor. The ability to activate B

cells in a highly specific fashion in the absence of intact T cells will also be employed to study the nature of signal transduction by B cells. Early response parameters such as PI hydrolysis and calcium flux will be studied in B cells activated either through lymphokine, antigen specific soluble helper factor, or combinations of such stimuli.

Publications:

Henkart P, Henkart M, Hodes R, Taplits M. Secretory processes in lymphocyte function. Bioscience Reports 1987;7:345-353.

Taplits MS, Henkart PA, Hodes RJ. T helper cell cytoplasmic granules: exocytosis in response to activation via the T cell receptor, J Immunol 1988; in press.

Asano Y, Nakayama T, Kubo M, Fujisawa I, Karasuyama H, Singer A, Hodes RJ, Tada T. Analysis of two distinct B cell activation pathways mediated by a monoclonal T helper cell. II. T helper cell secretion of interleukin 4 selectively inhibits antigen-specific B cell activation by cognate, but not noncognate, interactions with T cells. J Immunol 1988;140:419-426.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09267-06 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 Formerly Z01 CB 05110-05 I

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

T Cell Immune Function in Asymptomatic HIV Seropositive Patients

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

PI:	G. M. Shearer	Senior Investigator	EIB, NCI
	M. Clerici	Visiting Fellow	EIB, NCI
Others:	C. S. Via	Medical Staff Fellow	EIB, NCI
	M. Martin	Senior Investigator	LMM, NIAID
	F. Hakim	Senior Staff Fellow	EIB, NCI
	J. Berzofsky	Senior Investigator	MB, NCI

COOPERATING UNITS *(if any)*

R. A. Zajac and R. N. Boswell, HIV Unit, Lackland AFB, TX

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

A

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

Peripheral blood leukocytes (PBL) from HTLV-III antibody (+) and (-) donors were tested for IL-2 production and cell-mediated lympholysis (CML) to recall antigens (S+X) and to HLA allogeneic cells (ALLO). Among antibody (+) donors, approximately 50% failed to respond to S+X, whereas all responded to ALLO, except patients in the critical stages of AIDS, who responded to neither type of immunogen.

Using PBL from antibody (-) donors we demonstrated by cell fractionation techniques that S+X T cell responses are obliged to use $CD4^+$ T cells, whereas ALLO responses can be generated by either $CD4^+$ or $CD4^-$ T cells. Thus, the selective loss of S+X responses probably reflects the loss of $CD4^+$ cells during AIDS development.

By studying the T helper cell responses in 80 HIV^+ patients without AIDS, we have been able to subdivide Walter Reed Stage 1 patients into three subgroups: those that have lost no T helper cell (T_H) function; those that have lost only T_H to S+X; and those that have lost T_H function for both S+X and ALLO. We have not seen evidence for a defect in antigen-presenting cell function.

Project Description

Major Findings: This laboratory has been studying a small cohort of local volunteers (at 3-to-6 month intervals) that are HIV^+, as well as HIV^- volunteers with the same risk factors. These individuals include homosexual men and a few former i.v. drug users. We have recently gained access to PBL from a limited number of AIDS patients and patients with some AIDS-like symptoms. PBL from these groups of donors were sensitized in vitro to a battery of antigens that require CD4 T helper cells for IL-2 production and generation of CTL (i.e., influenza virus [FLU] and tetanus toxiod). We also activated PBL with stimuli that can use either $CD4^+$ or $CD8^+$ T helper cells (i.e., HLA alloantigens [ALLO] and PHA). Our findings indicate that approximately 50% of HIV^+ asymptomatic individuals did not respond to CD4-dependent antigens but did respond to antigens that can use the alternate CD8 T helper pathway either in generation of CTL or in IL-2 production. Many of the asymptomatic HIV^+ donors that failed to produce IL-2 when stimulated with influenza or tetanus had normal numbers of CD4 cells. The other 50% respond to all antigens tested. Among AIDS patients, approximately 50% failed to respond to CD4-dependent antigens, but responded to ALLO and PHA. PBL from the other AIDS patients failed to respond to any of the antigens tested.

These results indicate that functional T helper cell defects can occur prior to a loss of $CD4^+$ cells, which suggests that immune dysregulation in HIV^+ individuals is not solely attributable to a reduction in $CD4^+$ cells, and raises the possibility of a defect in antigen-presenting cell function.

Our study also indicates that many AIDS patients retain intact $CD8^+$ T cell function, including ability to produce IL-2 in response to HLA alloantigens. This finding raises the possibility that alloantigen-stimulated $CD8^+$ T helpers could provide help for CTL precursors that normally require $CD4^+$ T helpers. To test this possibility, PBL from HIV^+ individuals who were able to generate CTL to ALLO but not to FLU were cocultured with FLU plus ALLO. In the presence of costimulation with ALLO, the CD4-defective PBL generated CTL responses to FLU. This observation raises the possibility that AIDS patients who have lost $CD4^+$ T helper cell function but who have retained $CD8^+$ T helper and effector function could be immunized in vivo with allogeneic leukocytes plus antigenic determinants against opportunistic infectious agents to which AIDS patients are so susceptible, and possibly to HIV itself. This approach offers advantages over the injection of exogenous IL-2 because it should not have the adverse side effects of IL-2 injection in that it provides an in situ source of IL-2 as well as other lymphokines that might be important for the activation of T effector cells and B cells. The in vitro tests that we have developed to assess these functional pathways, including ALLO help for effector responses to viruses, provide a method for testing patients' T cells before attempting therapy.

In collaboration with Dr. Jay Berzofsky (NCI, NIH), we have tested PBL from approximately 60 HIV^+ individuals for proliferative responses and IL-2 production when stimulated with synthetic peptides from the gp120 envelope protein of HIV. We have also tested the proliferative responses of blood from healthy volunteers who were immunized to a vaccinia recombinant that expressed HIV antigens (Berzofsky et al., manuscript submitted for publication). We

found that PBL from the latter group of donors proliferated to the peptides. We found that PBL from approximately 30% of infected donors did not proliferate to the peptides, but did produce IL-2 when stimulated with the peptides. Very recent collaborative studies with Dr. M. Martin (NIAID) indicate that infant mice that express the human HIV gene do not exhibit changes in $CD4^+$ or $CD8^+$ T cell numbers, but do show elevated numbers of B cells. Spleen and lymph node cells from these mice exhibit defects in T lymphocyte function.

Publications:

Shearer GM, Singer A, Buller RM, Ambrus H, Morse HC. Importance of $CD8^+$ T helper cell function in AIDS (Letter to the Editor) J. Infect. Dis. 1988; in press.

Berzofsky JA, Bensussan A, Cease KB, Bourge JF, Cheynier R, Zirimwabagangabo L, Salaun J-J, Gallo RC, Shearer GM, Zagury D. Antigenic peptides recognized by T lymphocytes from AIDS viral envelope-immune humans. Nature 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09268-01 E

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Role of CD4 and CD8 Accessory Molecules in T Cell Function

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

PI:	A. Singer	Senior Investigator	EIB, NCI
Others:	S. McCarthy	Senior Staff Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	

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.)*

We have examined the role of CD4 and CD8 in MHC class recognition. In these studies, we have evaluated the ability of CD4 and CD8 molecules to function as signalling molecules for mature and developing T cells. Interestingly, we have found that CD4 function is related to TcR expression and appears to play an important signalling role in T cell differentiation. Indeed, there are a number of indications in these studies that CD4 and CD8 can both function as signalling molecules, but that they are not functionally identical molecules that merely differ in their ligand specificity for MHC class I or MHC class II determinants.

GPO 914-918

Project Description

Major Findings:

The concept that MHC class specificity expressed by T cells is determined by their cell surface expression of either CD4 or CD8 accessory molecules implicitly presumes that CD4 and CD8 molecules are functionally equivalent receptor molecules that differ only in their ligand specificity. Since activation of protein kinase C (PKC) by phorbol esters such as PMA can mimic receptor-mediated signalling and cause phosphorylation, internalization, and down-regulation of surface receptors, we examined if the responses of CD4 and CD8 to PKC activation were identical. To our surprise, we found that even though PMA activation of PKC induced rapid internalization and decreased cell surface expression of TcR on both $CD4^+$ and $CD8^+$ T cells, the responses of the CD4 and CD8 molecules themselves to PKC activation were markedly divergent. In contrast to CD8 molecules, CD4 behaved similarly to molecules comprising the TcR complex in response to PKC activation, in that CD4 molecules on mature T cells were internalized within minutes of PKC activation, resulting in markedly decreased cell surface expression of CD4. This CD4 response to PKC activation was not secondary to the effect of PKC on TcR molecules, as PKC activation also markedly reduced cell surface expression of CD4 on $CD4^+$ transfected L cells that did not express any TcR molecules. In contrast, cell surface expression of CD8 molecules on either mature $CD8^+$ T cells or $CD8^+$ transfected L cells was unchanged over at least a 5 hr time period, regardless of the dose of PMA used for PKC activation. Moreover, PMA induced PKC activation in $CD4^+CD8^+$ "double positive" thymocytes revealed, in a single cell population, a marked and rapid decrease in cell surface expression of CD4 but nearly unchanged cell surface expression of CD8 over 5 hours.

Because $CD8^+$ anti-class II Tk cells constitute the only "aberrant" T cell subset whose phenotype is "discordant" with its MHC specificity, we wished to compare their MHC differentiation requirements with those of "conventional" $CD4^+$ anti-class II Tk cells whose phenotype was "concordant" with their MHC specificity. Differentiation of all $CD4^+$ T cells, including $CD4^+$ anti-class II Tk cells, requires engagement of thymic MHC class II determinants as indicated by the arrest of $CD4^+$ T cell differentiation by in vivo administration of saturating amounts of anti-Ia antibodies. Consequently, we examined if differentiation of class II specific $CD8^+$ T cells was similarly arrested by in vivo anti-Ia blockade. Interestingly, we found that anti-Ia blockade did not interfere with the differentiation of $CD8^+$ anti-class II Tk cells even though, in the same animals, it arrested the differentiation of $CD4^+$ anti-class II Tk cells. These results demonstrated that differentiation of $CD4^+$ and $CD8^+$ anti-class II T cells differed fundamentally in their requirements for Ia engagement. Indeed, these results indicated that differentiation of $CD8^+$ T cells, even those whose receptors had specificity for MHC class II determinants, did not depend on engagement of thymic class II determinants, but, instead, either did not require MHC engagement at all or required engagement of MHC class I determinants. These observations have suggested to us that the MHC requirements for differentiation that developing T cells express may not reflect a requirement for TcR engagement, but rather may relect a requirement for MHC-induced cross-linking of CD4 or CD8 accessory molecules.

We examined the role of CD8 in T cell function by assessing the function of CD8 on precursor Tk (pTk) cells that would differentiate, upon activation, into mature Tk cells. Since anti-CD8 mAbs blocks recognition of class I target cells by most mature $CD8^+$ anti-class I Tk cells, it seemed reasonable to expect that anti-CD8 mAbs would similarly block $CD8^+$ pTk cell recognition of class I stimulator cells in the induction cultures and so inhibit anti-class I Tk cell responses. However, this turned out not to be the case. We found that addition of anti-CD8 mAbs to the induction cultures did not block generation of anti-class I Tk cells responses, but, paradoxically, frequently enhanced them. However, the anti-class I Tk cells that were generated in the presence of anti-CD8 mAbs were different than the anti-class I Tk cells that were generated in conventional induction cultures devoid of anti-CD8 mAb. Conventional Tk cells were overwhelmingly anti-CD8-sensitive in their ability to lyse class I targets, whereas novel Tk cells from cultures containing anti-CD8 mAbs were overwhelmingly anti-CD8-resistant. We found that the underlying mechanism responsible for anti-CD8 mAb induction of anti-CD8-resistant Tk cells involved: (a) multivalent cross-linking of CD8 molecules on the pTk cells, and (b) TcR engagement. We found that bivalent cross-linking of CD8 molecules on pTk cells did not lead to internalization and downmodulation of cell surface CD8, and resulted in blockade of pTk cell activation. In contrast, we found that multivalent cross-linking of CD8 molecules on pTk cells did lead to downmodulation of cell surface CD8 and did activate pTk cells to differentiate into anti-CD8-resistant Tk cells. Thus, these studies functionally demonstrate a signalling role for CD8 in Tk cell activation that requires multivalent cross-linking of CD8 to other CD8 molecules. In the light of these results, it is possible that activation of $CD8^+$ T cells requires two independent signals: one induced by CD8 cross-linking and one induced by TcR cross-linking.

Publications:

Golding H, Mizuochi T, McCarthy SA, Cleveland CA, Singer A. Relationship among function, phenotype, and specificity in primary allospecific T cell populations. Identification of phenotypically identical but functionally distinct primary T cell subsets that differ in their recognition of MHC class I and class II allodeterminants. J Immunol 1987;138:10-17.

McCluskey J, Singer A, Germain RN, Margulies DH. The role of CD4/L3T4 in T-lymphocyte function. Ann Inst Pasteur/Immunol 1987;138:150-157.

Mizuochi T, Tentori L, Sharrow SO, Kruisbeek AM, Singer A. Differentiation of Ia-reactive $CD8^+$ murine T cells does not require Ia engagement. Implications for the role of CD4 and CD8 accessory molecules in T cell differentiation. J Exp Med 1988;in press.

McCarthy SA, Kaldjian E, Singer A. Multivalent cross-linking of CD8 transduces an activation signal in developing cytolytic T lymphocytes. In: Kaplan JG, Green DR, eds. Cellular Basis of Immune Modulation. 1988;in press.

Kaldjian E, McCarthy SA, Sharrow SO, Littman DR, Klausner RD, Singer A. Nonequivalent effects of PKC activation by PMA on murine CD4 and CD8 cell surface expression. FASEB J 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09269-05 E

PERIOD COVERED
October 1, 1987 to September 30, 1988 formerly Z01 CB 05114-04 I

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Sequence Organization of Class I MHC Genes

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

PI:	Dinah Singer	Senior Investigator	EIB, NCI
Others:	Stuart Rudikoff	Senior Investigator	LG, NCI
	Leonardo Satz	Guest Researcher	U. Buenos Aires
	Rachel Ehrlich	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

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). We have established that this gene family contains only seven members, of which five have been isolated and their DNA sequences determined. It has been established that the sequence organization of the class I SLA genes is similar to that of other class I genes in other species. Within the family, it is possible to define at least three sub-families, based on their sequences. Homologies between the SLA genes range from 85% between two genes within a sub-family to 45% between sub-families. Gene conversion events appear to give rise to complex alterations, and to occur between sub-families. Physical mapping has established that the class I genes map within 500 kb on a single linkage group.

Isolation of cDNA clones has demonstrated that only 4 class I genes are expressed within swine PBL populations. Sequence analysis of these clones has established the locations of exons and further indicates a variety of post-transcriptional alternative splicing events occurs in these cells.

GPO 914-918

Project Description

Major Findings:

We have determined the DNA sequences of the five isolated class I SLA genes. From their sequence homologies, we have identified three sub-families. One sub-family consists of PD1, PD14 and PD7, which are between 81-85% homologous to one another. The other two sub-families contain only a single member. These genes, PD6 and PD15, are very divergent both from the PD1 sub-family (49-55%) and from each other (45%). By comparison with class I genes from other species, all of the members of the PD1 sub-family are composed of eight exons; in contrast, PD6 has only seven exons and PD15 has no discernible exons 6-8. In order to isolate the remaining two class I genes, we have screened a cosmid library, and are currently characterizing the isolated clones.

The physical linkage map of the class I locus is being investigated in two ways. Large scale mapping of the pig genome can be accomplished by either pulsed field or reverse field electrophoresis of DNA digests generated by restriction enzymes which cut infrequently within the genome. Blots derived from such gels are then hybridized using gene specific probes. We have isolated such specific probes for PD15 and PD6. Probes derived from PD1 identify all other sequences in the family. The SLA class I complex is contained within a single 500 kb segment, considerably smaller than the HLA class I complex, which has been estimated to span 2000 kb. Furthermore, PD6 maps to one end of the complex and is linked to PD15. An alternative approach, which will allow us to order individual genes more precisely, is isolation of cosmid clones, which we have just completed. Of the clones isolated, many have two or more genes contained within them. For example, PD14 and PD7 are located within a single cosmid clone, within 20 kb of each other. Thus, the spacing of class I SLA genes is similar to that of HLA genes. By combining the data obtained from the two approaches, it will be possible to generate a fairly accurate map of the gene family without necessitating extensive cosmid walking.

Because the splicing patterns of the SLA class I genes and their corresponding intron-exon boundaries had been inferred by comparison with class I genes of man and mouse, we isolated cDNA clones for the purpose of: 1) determining which of the seven genes could be expressed in vivo, and 2) the splicing patterns and 5′ and 3′ untranslated sequences. To this end, a cDNA library was constructed from RNA of peripheral blood lymphocytes and screened with both PD1 and PD6 probes. cDNA clones were isolated and grouped according to their hybridization pattern and restriction maps. Examples of each group were sequenced, yielding the cDNA sequences of PD1, PD14, PD7 and PD6. These cDNA sequences have confirmed our assignments of exon/intron junctions and have identified the sites of transcriptional initiation and termination.

Publications:

Ehrlich R, Lifshitz R, Pescovitz M, and Singer D. Isolation and characterization of a novel class I MHC Gene. J Immunol 1987;139:593-602.

Singer DS, Ehrlich R, Satz M, Frels W, Bluestone J, Hodes R, and Rudikoff S. Structure and Expression of class I MHC genes in the miniature swine. Vet Immunol Immunopathol 1987;17:211-221.

Singer D, Ehrlich R, Maguire J, Golding H, Satz M, Parent L, and Rudikoff S. Structure and organization of class I MHC genes in miniature swine. Molecular Biology of the MHC of Domestic Animal Species. 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09270-05 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05115-04 I

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.) (Name, title, laboratory, and institute affiliation)*

PI:	Dinah Singer	Senior Investigator	EIB, NCI
Others:	Rachel Ehrlich	Visiting Fellow	EIB, NCI
	Jean Maguire	Biotechnology Fellow	EIB, NCI
	Boaz Avidor	Visiting Fellow	EIB, NCI
	Jocelyn Weissman	Chemist	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	6.0	

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 investigate the mechanisms controlling the expression of a multi-gene family. The miniature swine has been chosen as an experimental model because there are only 7 members of the family. To address the question of the molecular regulation of the expression of class I MHC genes, two approaches have been taken: 1) the analysis of in vivo patterns of expression of each of the genes, both in situ and in transgenic mice; and 2) characterization of regulatory elements associated with these genes.

Analysis of in vivo patterns reveals that both shared and discrete regulatory mechanisms operate within the family. Thus, some genes are coordinately expressed, whereas others are not. In both cases, patterns of expression are actively regulated.

Regulatory sequences within one of the transplantation antigen genes have been identified by generating a series of 5′ end deletion mutants. A series of both positive and negative elements have been mapped. A novel negative regulatory element, which requires the presence of a positive element in order to function, has been identified. It has been further demonstrated that these elements function through the binding of trans acting factors. The binding sites of the trans acting factors have been mapped using both gel retardation assays and exonuclease protection.

GPO 914-918

Project Description

Major Findings:

Expression of individual class I genes is actively regulated: multiple genes within the family show similar patterns and responses, whereas other class I antigens, such as Qa and TL, have been classified as differentiation antigens because of their limited tissue distribution. We speculate that common mechanisms exist which coordinately regulate certain class I gene expression and discrete mechanisms exist which regulate expression of the differentiation antigens. In earlier studies, we demonstrated that introduction of one of the swine class I genes, PD1, into mouse L cells resulted in its regulated expression. Furthermore, interferon treatment of transfected L cells increased transcription of the PD1 gene, indicating that regulatory sequences were contained within the transfected DNA segment. In order to further define the regulation of PD1, we have examined its in situ patterns of expression as well as its expression in transgenic mice. In addition, we have constructed a variety of deletion mutants generated from the 5' flanking sequences which have allowed us to identify and define a series of regulatory elements. We are presently conducting parallel experiments with other members of this family in order to compare their regulation.

We have undertaken a molecular dissection of PD1 with the aim of identifying functional regulatory DNA sequence elements. To date, our studies have focussed primarily on the 1.1 Kb of 5' flanking region contained within the transgene. Using a limited series of 5' deletion mutants, ligated to the reporter gene CAT, we have identified the transcriptional promoter, the interferon response element (IRE) and an array of positive and negative regulatory elements. Of particular interest is the existence of a novel negative element. Removal of this element from the 5' flanking segment increases expression from the PD1 promoter by 5-10 fold. Introduction of the silencer segment adjacent to the heterologous viral SV40 promoter similarly reduces its efficiency of transcription. However, this negative effect is observed only if the SV40 promoter has its enhancer element associated with it. In the absence of the viral enhancer, the PD1 silencer does not function. In vivo competition experiments, employing various combinations of enhancers and the PD1 silencer have demonstrated that trans acting factors mediate the function of these elements. In particular, a PD1 enhancer shares at least some common elements with the SV40 enhancer. Furthermore, the trans acting factor(s) binding to the silencer element does so only in the presence of an enhancer and its cognate binding factors. Although the molecular mechanisms by which the regulatory elements and their factors function is not known, our data suggest a model in which the negative and positive regulatory elements each define an independent binding site .

Functional analysis of the PD1 5' flanking region has documented the existence of at least three enhancers and a silencer (see above). Inspection of the DNA sequence of the PD1 5' flanking region revealed no striking homologies with known regulatory elements from other gene systems, other than the interferon responsive element, the H2TF1 binding site, and enhancer B. In

order to attempt to map the binding sites of PD1 trans acting factors, we have employed an exonuclease protection assay. This assay consists of introducing the bacterial lac operon adjacent to a test DNA fragment. Addition of lac repressor, which binds tightly to the operator, creates a strong exonuclease stop site which serves as a reference point. Following addition of nuclear extract, the DNA-protein complex is digested with exonuclease, generating new stops at the sites of factor binding. Using this assay which allows precise mapping of multiple binding sites, we have been able to identify 6 exonuclease stop sites, none of which map to the DNase I site. Four of these sites are independent of the presence of nuclear extract, and suggest the presence of secondary structure within the segment. Two sites are specific for the presence of nuclear extract. One site falls within the DNA segment known to contain the silencer element; the other is contained within the DNA segment known to contain an enhancer. Further characterization of these binding sites is now being done by the gel retardation assay, using fragments spanning the mapped regions.

The patterns of expression of four other class I genes have been examined. One, PD14, encodes a classical transplantation antigen and is coordinately expressed with PD1. A second gene, PD7, has been shown to have precisely the same pattern of expression as PD1, but at markedly reduced RNA levels relative to PD1 in all tissues. We speculate that PD7 may be a third transplantation antigen, analogous to HLA-C. Transfection of PD7 into mouse L cells results in a very low level of transcription and commensurately low level of cell surface expression. Removal of 3 kb of 5′ flanking sequence from PD7 increases its level of transcription. A third gene, PD6, displays patterns of expression distinct from PD1. Although PD6, like PD1, is expressed in pigs at the highest levels in lymphoid tissues, unlike PD1, it is preferentially expressed in T cells, relative to B cells, and is not expressed at all in the thymus. The fourth gene, PD15, is not detectably expressed in any adult tissue nor in transfected L cells, and is considered to be a pseudogene.

Publications:

Ehrlich R, Lifshitz R, Pescovitz M, and Singer, D. Isolation and characterization of a novel class I MHC gene. J Immunol 1987;139:593-602.

Singer D, Ehrlich R, Satz M, Frels W, Bluestone J, Hodes R, and Rudikoff S. Structure and Expression of class I MHC genes in the miniature swine. Vet Immunol Immunopathol 1987;17:211-221.

Ehrlich R, Maguire J, and Singer D. Identification of negative and positive regulatory elements associated with a class I major histocompatibility complex gene. Mol Cell Biol 1988;8:695-703.

Singer DS, and Ehrlich R. Identification of regulatory elements associated with a class I MHC gene. Current Topics in Microbiology 1988;in press.

Singer D, Ehrlich R, Maguire J, Golding H, Satz L, Parent L, and Rudikoff S. Structure and organization of class I MHC genes in miniature swine. Molecular Biology of the MHC of Domestic Animal Species 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09271-05 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05116-04 I

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

Graft-versus-Host Disease in Allogeneic Bone Marrow Transplantation

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

PI:	Ronald E. Gress	Senior Investigator	EIB, NCI
Others:	Robert Moses	Biotechnology Fellow	EIB, NCI
	Raphael Hirsch	Medical Staff Fellow	EIB, NCI
	Hirotoshi Nakamura	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

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.)*

Graft-versus-host disease (GvHD) is dependent in its generation on the presence of immune competent T cells in the donor marrow with specificity for allogeneic antigens of the host. The activation of these cells in the host after infusion and the resultant tissue injury is clinically recognized as GvHD. One approach to preventing GvHD is the removal of these T cells from the marrow inoculum. We have pursued T cell depletion of human marrow for the purpose of preventing GvHD in marrow transplantation across MHC differences in man by the use of monoclonal antibodies specific for cell surface molecules unique to T cells in combination with complement. Two such antibodies have been derived, one with specificity for CD2 (sheep red blood cell receptor) and one which defines a novel T cell determinant. The latter antibody precipitates a 92 KD molecule and a predominant 45 KD band under non-reducing conditions and reducing conditions, respectively. These two antibodies, in combination with CD7 and CD5 specific antibodies, have been used to carry out studies in human bone marrow T cell depletion. To quantitate T cells remaining in marrow after such depletions, a limiting dilution assay was developed which detects T cells at a level of one T cell per 10^5-10^6 marrow cells. A bank of cryopreserved marrows depleted of T cells by a combination of monoclonal antibodies and complement, with the extent of that depletion assessed by limiting dilution assay, has been established and a clinical protocol developed to assess the feasibility of utilizing these marrows in the treatment of aggressive malignancy with marrow ablative combination chemotherapy and radiotherapy.

GPO 914-918

Project Description

Major Findings:

The primary approach taken in these studies of T cell depletion of human marrow has been by antibody and complement; alternative approaches such as the use of immunotoxins, elutriation, and immune rosetting have also been evaluated. Initial studies with antibody and complement established conditions for optimal complement mediated lysis and showed that individual antibodies differed in their ability to effect lysis in the presence of complement. A combination of antibodies was superior to single agents not in the extent of depletion, but in reproducibility from treatment to treatment. In addition to two antibodies available from other sources (CD 7 and CD 5 specific), neither of which was adequate to mediate pan T cell depletion effectively, we derived additional monoclonal antibodies with screening by complement mediated lysis. Two antibodies of interest were isolated. One was CD2 specific and has been utilized in these studies for T cell depletion and in investigations of the role of the LFA-2 accessory molecule in T cell function. It detects an epitope region distinct from that detected by OKT11. The second antibody is specific for a novel determinant on T cells. The determinant for which this antibody is specific is expressed by cells of hematopoietic origin, is confined to T cells, and is concordant with the expression of CD5 and CD3. Immunoprecipitation demonstrates a 92 KD molecule under non-reducing conditions and a predominate 45 KD band under reducing conditions. Comparisons of expression on a series of T cell lines, including a line deficient in the expression of T cell receptor, distinguished the determinant defined by this antibody from those detected by known anti-T cell monoclonal antibodies.

The number of donor marrow T cells necessary for the generation of GvHD is on the order of 0.1% in the mouse or 1 x 105/kg in man. Assays commonly used for the quantitation of residual T cells after depletion such as proliferative responses to mitogens (PHA) or alloantigens (MLR) or analysis by FACS detect on the order of 1% T cells and so are insufficient in sensitivity. A limiting dilution assay was therefore developed based on the clonogenic potential of peripheral human T cells. The readout is cell growth; the sensitivity of this assay is sufficient to detect one T cell in 10^5-10^6 marrow cells and the specificity has been confirmed by a variety of techniques. This limiting dilution assay has been essential to the monitoring of T cell depletion from human marrow. With this assay, the depletion regimen was refined and different methods of marrow harvest evaluated with respect to influence on final marrow T cell content. Surgical resection of marrow was superior to conventional aspiration as a method of harvest. One consideration in the clinical use of surgically resected marrow is marrow cell yield. The final yield in a series of harvested marrows (after treatment to remove T cells) was 64% of the Ficoll population for a final mean total cell number of 9.2×10^9 cells per donor. The residual T cell content was less than one T cell per 10^5 marrow cells which represented, in each case, greater than a three log depletion of T cells. Progenitor cell (CFU-C and BFU-E) recovery was 42-100%. These and similarly prepared marrows have been cryopreserved as a "universal donor bank".

Publications:

Lucas P, Gress RE. Specific selection of lymphocyte subpopulations by avidin-biotin immune rosetting. J Immunol Methods 1987;99:185-190.

Lucas P, Quinones RR, Moses RD, Nakamura H, Gress RE. Alternative donor sources in HLA-mismatched marrow transplantation: T cell depletion of surgically resected cadaveric marrow. Bone Marrow Transplantation 1988;3:211.

Sakamoto K, Shimada S, Popitz-Bergez FA, Pennington LR, Pescovitz MD, McDonough M, Katz SI, Sachs DH, Gress RE. Bone marrow transplantation in miniature swine. III. GVHD and the effects of T cell depletion of marrow. Transplantation 1988;45:869.

Quinones R, Gress RE. A new anti-human T cell monoclonal antibody with specificity for a novel determinant. Transplantation 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09272-05 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05118-04 I

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

Regulation of Immune Response to Tumor Cells and Alloantigens

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

PI:	C. C. Ting	Senior Investigator	EIB, NCI
Others:	M. E. Hargrove	Microbiologist	EIB, NCI
	Y. S. Yun	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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.)*

1. Anti suppression T cells (TAS). T cell differentiation factor (TCDF) induced the generation of TAS in allogeneic mixed lymphocyte culture. TAS specifically abrogated the suppressive effect of TS (suppression T cells) which suppress CTL activity. Generation of TAS was dependent on CD4 and Thy 1 molecules but was not affected by antibodies against CD8 or class II MHC determinants, and thus TAS were different from contra suppressor cells or T helper cells. TAS may represent a new class of immunoregulatory cells that regulate the cytotoxic activity of CTL.

2. Asialo GM1 as an accessory molecule for CTL - mediated cytotoxicity. The blocking by αASGM1 was largely dependent on the expression of ASGM1 on effector cells. Furthermore, all $ASGM1^+$ CTL exerted different degrees of NK- or LAK-like cytotoxicity, whereas the $ASGM1^-$ CTL displayed more restricted specificity against appropriate targets. Hence, the expression of ASGM1 on CTL determines both the specificity and the cytotoxic activity of the cytotoxic reactions.

3. Effect of αCD3 on lymphokine-activated killer cell activity. Antibody against the ε chain of T3 complex on T cells (αCD3) could augment the effector function of the cytotoxic activity of LAK cells, and could induce the generation of killer cells (CD3-AK) through the triggering of endogenous production of lymphokines by αCD3. The cytotoxic activity of the αCD3-induced killer cells (CD3-AK) generally paralleled that of the conventional LAK cells. In contrast to LAK cells which only survived in culture for 6-10 days, the CD3-AK cells could be maintained in vitro in active growth for 3-5 weeks with high levels of anti-tumor cytotoxic activity. Thus the CD3-AK may be an ideal candidate for use in adjuvant adoptive immunotherapy of cancer.

Major Findings:

I. Regulation and Control of Immune Responses:

1. T cell-differentiaiton factor-induced late help through the generation of anti-suppressor T cells (TAS): We previously demonstrated that T cell differentiation factor (TCDF) worked in conjunction with IL-2 to induce the differentiation of cytotoxic T cell (CTL). We later found that adding TCDF to bulk-cultured CTL could sustain their growth and cytotoxic activity for at least 15-30 days, whereas the regular CTL lost their cytotoxic activity and ceased to grow in 7-10 days. Since the termination of cytotoxic activity of bulk-cultured CTL was attributed to the generation of suppressor T cells (TS), experiments were performed to determine whether TCDF could prevent TS generation. We found that TCDF did prevent the MLC (mixed-lymphocyte culture) induced suppression. In determining the serologic phenotype of the effectors involved in the MLC-induced suppression, we found that, first, the suppressor cells were shown to be $Thy1^+$ and $CD8^+$ and, thus were the classic TS. Second, after depletion of $CD8^+$ cells in TCDF-supplemented MLC, the remaining cells contained suppressor activity, indicating TCDF did not prevent TS generation. Third, depletion of $CD8^+$ cells in TCDF-supplemented MLC did not result in the increase of alloreactive CTL generation in second MLC, indicating that TCDF did not seem to induce extra helper T cells. Therefore, it appeared that anti suppressor T cells (TAS) were induced by TCDF. The mixing experiments confirmed that TAS were truly induced in TCDF-supplemented MLC. Mixing TAS with TS specifically abrogated the TS activity directed against the same H-2 haplotype. The generation of TAS did not require $CD8^+$ cells and thus TAS were different from contra suppressor cells. Generation of TAS was completely blocked by αCD4 antibody but was unaffected by antibodies against class II MHC framework determinants, therefore TAS were different from $CD4^+$ helper T cells that were induced in the context of recognizing class II MHC determinants. TAS may represent a new class of immunoregulatory cells that regulate the cytotoxic activity of CTL.

2. Glycolipid asialo GM1 as an accessory molecule for mediating CTL cytotoxic reactions. In previous reports we showed that asialo GM1 (AsGM1) was expressed on a majority of $CD8^+$ cloned CTL. Purified AsGM1 or antibody against AsGM1 blocked the cytotoxic reactions mediated by $ASGM1^+$ CTL. We further found that the blocking of cytotoxicity by αAsGM1 was largely dependent on the expression of ASGM1 on the effector cells in a dose-dependent fashion. The expression of AsGM1 on target cells had little effect in the blocking of cytotoxic reactions by either αAsGM1 or AsGM1. We also found that the expression of AsGM1 and CD4 molecules on activated T cells were mutually excluded. All the cloned $CD4^+$ CTL or T helper cells and $CD4^+$ Con A-induced lymphoblasts were $AsGM1^-$. However, CD4 and AsGM1 could be co-expressed on resting T cells. All the $AsGM1^+$ CTL derived from bulk-cultured CTL or CTL clones exerted different degrees of NK- or LAK-like cytotoxicity. The $AsGM1^-$ CTL displayed more restricted specificity against appropriate targets. Hence, the expression of AsGM1 on CTL not only determines their cytotoxic activity, it may also modulate the specificity of the cytotoxic reactions.

II. Tumor Immunology:

1. Augmentation by αCD3 of the lymphokine-activated killer (LAK) cell-mediated cytotoxicity. The CD3 molecule of TCR (T cell receptor) was found to be involved in signal transduction of T cell activation. Experiments were performed to determine whether a correlation exists between CD3 molecules and LAK cell cytotoxic activity. We found that antibody against the ε chain of CD3 complex (αCD3, 145-2C11) augmented the effector function of LAK cell-mediated cytotoxicity. The αCD3-augmented killing was only seen with tumor targets, and there was no increased killing against Con A-induced lymphoblasts. For some targets, the αCD3-augmented LAK killing was Fc receptor (FcR) dependent as evidenced by the ability of αFcR mAb 2.4G2 to inhibit, and for others it was not inhibited. The αCD3-augmented killing did not correlate with the FcR expression on targets as defined by 2.4G2. The LAK cells were both $CD8^+$ and $CD8^-$, but the LAK cells involved in αCD3-augmented killing were exclusively $CD8^+$ cells. These results suggested that a relationship might exist between the CD3 complex and the cytotoxic activity of a subpopulation of $CD8^+$ LAK cells.

2. Heterogeneity of the long-term cultured αCD3-induced activated killer cells (CD3-AK). The αCD3 could activate $CD3^+$ precursors to induce lymphokine production and generation of cytotoxic effectors which were designated as CD3-AK. Similar to LAK cells, the CD3-AK were induced by lymphokines and selectively killed tumor targets. Once the CD3-AK were fully activated, their cytotoxic activity no longer required the presence of αCD3. In contrast to conventional LAK cells which only survived 7-10 days, the CD3-AK cells could be maintained in culture with active growth for 3-5 weeks. We have recently obtained a cytotoxic CD3-AK cell line which is in culture for more than 4 months. The newly induced CD3-AK cells (1-2 wk) were predominantly $CD3^+$ $CD8^+$ cells. After culturing for 3-4 weeks, $CD8^-$ cells emerged, suggesting the heterogeneity of CD3-AK cells.

3. Requirement of multiple cell types and multiple lymphokines in the generation of LAK cells and CD3-AK cells. In addition to IL2, we found that CCDF (cytotoxic cell differentiation factor) and IL4 synergized with IL2 to induce LAK cells and/or CD3-AK cells. Subpopulations of T cells ($CD4^+$ T cells) or accessory cells (Ia^+) were needed in the absence of exogenous lymphokines. The requirement of FcR^+ accessory in the generation of αCD3-induced proliferative response is different from αCD3-induced cytotoxic response. It appeared that multiple cell types and multiple lymphokines were involved in the generation of an efficient LAK or CD3-AK cell response.

4. Relationship between LAK cell activation and protein kinase C (PKC) activity. Our preliminary experiments showed that there were PKC-dependent pathway and PKC-independent pathway in the generation of LAK cells and CD3-AK cells. They showed different degrees of susceptibility to the inhibitory effect of H7 (a PKC inhibitor) or prolonged treatment with PMA which could deplete PKC. These findings suggested that TCR-T3 complex might play a role in the activation of subsets of LAK cells.

Proposed Course of Research:

1. To study the role of antisuppressor T cells in the regulation of LAK cell response.

2. Cellular and lymphokine regulation of LAK cell response. To determine the effect of different cell types ($CD4^+$ or $CD8^+$ T cells, Ia^+ accessory cells or FcR^+ accessory cells) and various lymphokines (IL4, IFN_S, etc) on LAK cell generation.

3. Mechanism for αCD3-induced cytotoxicity. To determine the role of protein kinase C, inositol phosphate, and T cell receptor (α, β vs γ, δ) expression on CD3-AK response.

4. In vivo anti-tumor activity of short-term and long-term cultured CD3-AK cells. The animal tumor models to be used in these experiments are melanoma (JB/MS) in syngeneic C57BL/6 mice, and mastocytoma (P815) in DBA/2 mice.

Publications:

Ting CC, Hargrove ME. Generation of antisuppressor T cells for alloreactive cytotoxic T lymphocytes. J. Immunol. 1987;139:3964-72.

Hargrove ME, Ting CC. Asialo GM1 as an accessory molecule determining the function and reactivity of cytotoxic T lymphocytes. Cellular Immunol. 1988; 112:123-34.

Ting CC, Hargrove ME, Yun YS. Augmentation by anti-T3 antibody of the lymphokine-activated killer cell-mediated cytotoxicity. J. Immunol. 1988; in press.

Yun YS, Hargrove ME, Ting CC. Heterogeneity of long-term cultured activated killer cells induced by anti-T3 antibody. J. Immunol. 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09273-01 E

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

T Cell Differentiation and Repertoire Selection

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

PI:	A. Singer	Senior Investigator	EIB, NCI
Others:	E.W. Shores	Guest Researcher	EIB, NCI
	T. Mizuochi	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.35	1.35	

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 examined thymic influence on the MHC-specificity expressed by functionally and phenotypically distinct T cell subsets. These studies have indicated a hitherto unappreciated role of nominal self antigens in thymic selection of the developing T cell repertoire. Studies on thymocytes from genetically defective scid mice have suggested a relationship between TcR gene arrangement and expression of CD4/CD8 accessory molecules in thymocyte differentiation. Studies with thymic organ cultures have revealed that it is possible to generate functionally competent T cells from fetal liver stem cells in vitro.

Project Description

Major Findings:

Anti-class I $CD4^+$ Th cells recognize processed foreign class I MHC determinants presented by self-class II determinants. Because of their dual MHC specificity, it was of interest to determine which class of MHC molecule functioned as their selection specificity during intra-thymic differentiation. Specifically, we wished to determine whether precursors to $CD4^+$ $H\text{-}2^b$ Th cells that recognize an antigenic complex composed of K^{bm6}+IA^b interacted with thymic class I or thymic class II MHC determinants during differentiation. As would be expected for the differentiation of any IA^b-restricted $CD4^+$ Th cell, we found that it was necessary for the thymus to be phenotypically IA^b. Surprisingly, however, we also found that the ability of IA^b-restricted $CD4^+$ Th cells to recognize K^{bm6} foreign determinants as nominal antigen was also dependent upon the MHC class I phenotype of the thymus. That is, to generate Th cells specific for the antigenic complex K^{bm6}+IA^b, it was necessary for the thymus to express the complex K^b+IA^b. These results are the first indication that the developing Th cell repertoire is not shaped by the specificity of thymocytes for naked Ia determinants, but rather that it is based on their specificity for complexes of self antigens presented by self-Ia in the thymus.

In order to examine the general relationship between TcR expression and T cell differentiation, we have examined a genetically defective mouse strain. Mice with severe combined immune deficiency (scid), lack both receptor bearing T cells and receptor bearing B cells. It is thought that this genetic defect results from a deficiency in the recombinase enzymes necessary for receptor gene rearrangements, making it very difficult for the lymphocytes in these animals to express any antigen receptors. As a result, these animals represent an excellent model for examining how far T cell differentiation can progress in the absence of TcR expression, and for indicating which steps are in fact dependent on TcR. In normal T cell ontogeny, T cells can be characterized by their expression of CD4 and CD8 accessory molecules. Thus, the kinetics of appearance of thymocyte subsets is: $CD4^-CD8^-$ -> $CD4^+CD8^+$ -> $CD4^+CD8^-$, $CD8^+CD4^-$. The $CD4^+CD8^+$ double positive subset consists of approximately equal numbers of TcR^+ and TcR^- cells, suggesting that TcR expression is not necessary for thymocytes to become $D4^+CD8^+$. Consequently, the expectation was that, in scid mice, thymocyte development would proceed through the generation of double positive cells. To our surprise, we have found that $Thy1^+$ thymocytes from most scid mice contain only $CD4^-CD8^-$ (double negative) TcR^- cells. These cells are $IL\text{-}2R^+$ and Ly1 dull, and so are similar to double negative cells from the thymi of normal mice. What is surprising from these results is that, in the absence of TcR rearrangement, T cell differentiation does not proceed beyond double negative cells, suggesting that CD4/CD8 expression during T cell differentiation may directly or indirectly depend upon rearrangement of TcR genes.

To better dissect the intra-thymic events involved in T cell differentiation and selection, it is potentially advantageous to study these events in vitro. It is possible to place fragments of embryonic thymus into organ culture and have T cell differentiation proceed as indicated by expression of

physical markers. Indeed, it is also possible to construct in vitro chimeras by co-culturing fetal liver, as a source of stem cells, with lymphocyte depleted thymic fragments. In these in vitro cultures, fetal liver derived stem cells migrate into the thymic fragment and begin differentiating into T cells. In collaboration with Dr. Dom DeLuca, University of So. Carolina, we have extensively assessed the T cells that arise from such in vitro chimeras for function and for specificity. We have found that T cell populations arising in these in vitro chimeras contain both Tk cells and IL-2 secreting Th cells, demonstrating that T cells can acquire function in these in vitro cultures. However, the specificity these T cells express is highly unusual in that they react, both cytolytically as well as by secretion of lymphokine, to both allogeneic and syngeneic stimulator cells.

Publications:

Singer A, Munitz TI, Gress RE. Specificity of thymic selection and the role of self antigens. Transplant Proc 1987;19:107-110.

Mizuochi T, Singer A. Intrathymic differentiation and tolerance induction of lymphokine secreting $Lyt2^+$ T helper cells. J Immunol 1988;140:1421-1424.

Singer A. Commentary: Experimentation and thymic selection. J Immunol 1988;140:2481-2483.

Mizuochi T, Tentori L, Sharrow SO, Kruisbeek AM, Singer A. Differentiation of Ia-reactive $CD8^+$ murine T cells does not require Ia engagement. Implications for the role of CD4 and CD8 accessory molecules in T cell differentiation. J Exp Med 1988;in press.

McCarthy SA, Singer, A. T-lymphocyte associative recognition. In: Litwin, Scott, Flaherty, Reisfeld, Marcus, eds. Human Immunogenetics: An Advanced Text. 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09274-05 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05120-04 I

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)*

P.I.:	K. Kelly	Senior Investigator	EIB, NCI
Others:	S. Irving	Guest Researcher	EIB, NCI
	K. Gunter	Biotechnology Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

Ulrich Siebenlist, Laboratory of Immune Regulation, NIAID, NIH

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.8	2.8	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 goal of this project is to broaden our understanding of the physiology and regulation of early events in T cell activation. Lymphocyte metabolism and effector function expression are regulated by antigen/mitogen and lymphokine binding to cell surface receptors. We are investigating the consequences of mitogen mediated signals by isolating and characterizing genes which are transcriptionally regulated by these events. We have constructed a subtracted cDNA library enriched for genes that are transcriptionally induced within four hours after stimulating peripheral blood T cells with PHA and PMA. Utilizing the library in combination with labelled subtracted probes (activated T cells cDNA minus resting T cell mRNA), we have isolated over 60 novel cDNA clones which represent the immediate response of resting human peripheral blood T cells when activated by mitogen to proliferate. This primary response is highly complex, both in terms of the number of inducible genes as well as the diversity of their regulation. Although a majority of the genes involved are shared with the activation response in normal human fibroblasts, a significant number are more restricted in their tissue specificity and thus may be encoding or effecting the differentiated functions of activated T cells. Surprisingly many inducible genes are inhibited by the immunosuppressive drug cyclosporin A, which is known to repress the transcription of several lymphokine genes. In addition, a subset of inducible genes are stimulated to constitutive expression in T cell clones infected with the immortalizing human retrovirus, HTLV I. Such abnormally-expressed genes may play a role in the deregulated growth of such cells.

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

Project Description

Major Findings:

The goal of this project is to broaden our understanding of the physiology and regulation of mitogen-induced activation. To this end we are isolating and characterizing genes that are induced rapidly following mitogen stimulation of human peripheral blood (PB) T cells. Primary gene transcriptional events, defined by their early kinetics and lack of dependence on protein synthesis, include proto-oncogenes (e.g. c-myc), lymphokines (e.g. IL-2, γ-IFN, GM-CSF), and lymphokine receptors (IL-2 receptor), all of which are thought to have significant effects upon T cell proliferation and/or regulation of an immune response. Because the number of genes inducible by a mitogenic signal is unknown but likely to exceed the reported genes, we sought to thoroughly characterize the initial response to an activation signal by cloning a maximal number of induced genes. At least some of the immediately induced genes would be expected to initiate and thus regulate the cascade of molecular events that follow mitogenic activation.

We have used a cloning technique that provides maximal sensitivity in detecting genes encoding mRNAs expressed even in low copy number. That is, we have employed the techniques of subtractive cDNA cloning coupled with using a labelled subtracted probe to select genes that are transcriptionally induced in PB T cells within four hours following the addition of PHA and PMA in the presence of cycloheximide. We have isolated in excess of 60 unique genes that are unrelated as determined by cross-hybridization analyses. Although known induced genes such as c-myc and IL-2 receptor are included within these genes as expected, greater than 90 percent of the clones represent previously undescribed induced genes. The clones demonstrate heterogeneity with regard to kinetics of induction, ranging from 30 minutes to 4 hours, and levels of expression, encompassing less than 10 to greater than 100 induced copies per cell. The number of induced genes is unexpectedly large, suggesting that the initial response to a mitogen is complex and likely to involve many cellular functions beyond a direct role in proliferation.

Analyses of the large number of induced clones that have been isolated offer the opportunity to begin elucidating the complexity of mitogen-induced responses. However, it is important to establish criteria to decide which clones should be the first subjected to detailed investigation. Two important broad areas of interest are the physiological functions of induced genes and the spectrum of regulatory mechanisms controlling induced gene expression.

An important step in the investigation of gene function is primary structure determination. We currently are concentrating such analyses on those induced genes that are T cell-specific, as such genes most likely encode specialized "non-housekeeping" functions that could relate to either proliferation or differentiation. Four of 16 clones assayed to date demonstrate a tissue-restricted phenotype, while the remaining 12 are expressed in both mitogen-activated lymphocytes and fibroblasts. Currently, we have completed the primary structural analysis of 3 genes, pAT 464, 744, and 555, two of

which (pAT 464 and 555) are greater than 90% homologous. All three genes encode small proteins that most likely are secreted products and therefore may be previously undescribed lymphokines.

Our data suggests that the initial biochemical response leading to mitogen-induced gene induction is complex, and therefore we expect that various distinct pathways mediate the induction of groups of genes within the large family we have isolated. Such groups of genes most likely share common DNA regulatory elements. One approach to defining regulatory families of induced genes is to employ specific and singular stimuli that affect distinct intracellular secondary signals. Such an analysis of nine novel induced genes has demonstrated the following: 1) Fully mitogenic agents that stimulate multiple intracellular secondary signals such as anti-T3 mAbs induce all nine novel genes assayed. 2) By utilizing agents that stimulate a partial complement of intracellular secondary signals and must be combined with additional stimuli to mediate mitogenesis, we have distinguished within the 9 genes assayed 5 groups that demonstrate unique patterns of responses. Interestingly, pAT 464 and 744 referred to previously as potential lymphokines are regulated similarly to IL-2 and distinctly from the other genes assayed.

An additional approach to defining different activation requirements makes use of the immunosuppressive drug, cyclosporin A (CsA). Because CsA has been shown to selectively inhibit the induction of some genes, mostly lymphokines, but not others, it appears that CsA acts upon some activation pathways while leaving others intact. We have investigated the extent of CsA action on the expression of a large number of inducible genes assayed in the T cell line Jurkat. Of the twenty-two genes assayed that are inducible in Jurkat cells, ten were inhibited by CsA. CsA appears to inhibit induction via a transcriptional mechansim as determined by nuclear run-on assays. Thus, these ten genes likely share a common activation pathway(s) or element(s) necessary for induction. However, the CsA-regulated group does not specifically correlate with a mitogen-response defined family, suggesting considerable complexity and heterogeneity in the mechanisms leading to mitogen-stimulated gene induction. In addition, because many CsA-regulatable genes in Jurkat also are induced in mitogen-treated human fibroblasts, we are in a unique position to address the tissue specificity of CsA action on gene induction. Several genes that are mitogen-induced and inhibited by CsA in Jurkat cells are induced in mitogen-treated fibroblasts but unaffected by CsA at the steady state mRNA and transcriptional level. The above results suggests that CsA effects a T cell specific regulatory element that is required for gene induction in T cells but not fibroblasts.

By analogy to known activation-induced genes such as c-myc and IL-2 receptor that are thought to play a causative role in various lymphoid neoplasias, it seems likely that abnormal expression of some mitogen-induced genes described here may similarly act to initate or maintain transformed growth. To investigate this possibility, we have assayed the expression of several mitogen-induced genes in adult T cell leukemia derived cell lines and in normal human T cell clones that are immortalized following HTLV I infection. Interestingly, fifteen induced genes that are transiently expressed in

antigen or mitogen treated normal T cell clones are expressed constitutively at high levels in HTLV-I infected clones and in ATL-derived cell lines. It is reasonable to think that some induced genes whose expression is upregulated in constitutively-growing, transformed T cells may play an integral part in regulating cellular proliferation.

Publications:

Kelly K, Underwood B. Cell growth associated regulation of c-myc and c-fos in normal human T cells. Adv Exp Med Biol 1987;213:241-247.

Trepel JB, Colamonici OR, Kelly K, Schwab G, Watt RA, Sausville EA, Jaffe EJ, Neckers LM. Transcriptional inactivation of c-myc and the transferin receptor in dibutyryl cyclic AMP-treated HL-60 cells. Mol Cell Biol 1987;7:2644-2648.

Siebenlist U, Bressler P, Kelly K. Two distinct mechanisms of transcriptional control operate on c-myc during differentiation of HL-60 cells. Mol Cell Biol 1988;8:867-874.

Kelly K, Siebenlist U. Mitogenic activation of normal T cells leads to increased initiation of transcription in the c-myc locus. J Biol Chem 1988;263:482-486.

Kelly K. T cell activation. In: Melchers F, Potter M, eds. Mechanisms of B cell neoplasia. 1987;424-433.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09275-01 E

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

In Vivo Study of MHC-Specific T Cells

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

PI:	A. Singer	Senior Investigator	EIB, NCI
Others:	A. Rosenberg	Guest Researcher	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	

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 has attempted to apply our understanding of the cellular mechanisms involved in in vitro anti-MHC responses to in vivo transplantation responses. In studying skin allograft rejection, we have identified the phenotype, specificity, and interaction capabilities of the T cells able to initiate and effect in vivo rejection responses. Using allophenic skin grafts, we found that the effector mechanism of skin graft rejection is itself antigen-specific, and that defects in Th cell function result in longterm retention of skin allografts despite the presence of antigen-specific effector cells. In addition, we found that expression of class I and class II MHC antigens on skin epidermal cells is regulated, and the pattern of MHC expression importantly influences the cellular immune mechanisms that initiate and effect graft rejection responses.

Project Description

Major Findings:

We have been studying transplantation immunity as the in vivo analogue of the anti-MHC responses we had been examining in vitro. Consequently, our initial focus was on determining the phenotype, function, and specificity of the T cells that initiate and mediate in vivo rejection responses. We chose to study the rejection of skin allografts because skin allografts trigger the most potent of all in vivo rejection responses, and because they are convenient to assess and follow.

We began our studies by trying to identify, by phenotype and function, the T cell subset that was responsible for determining the rejection rate of skin allografts across a well-defined, MHC class I disparity created by 2-5 amino acid changes in the K^b molecule. We engrafted H-2^b mice with tailskin from various K^b mutant mouse strains and determined their rejection rates. We found that there existed a highly reproducible hierarchy of rejection rates, such that bm1>bm3=bm10>>bm6. This hierarchy of rejection rates correlated perfectly and uniquely with the precursor frequencies in H-2^b mice of $CD8^+$ IL-2 secreting Th cells specific for the various K^b mutant determinants, such that $CD8^+$ Th cells specific for bm1 were most frequent, followed by anti-bm3 and anti-bm10, with anti-bm6 virtually undetectable. These results provided the first indication that $CD8^+$ IL-2 secreting Th cells functioned in vivo and were the limiting cell type determining the rejection rate of K^b mutant class I disparate skin allografts. In order to directly assess the importance of Th cells in in vivo rejection responses, we simultaneously engrafted mice with two skin allografts. One graft, considered the indicator skin graft, expressed only the target antigen of interest; the second graft, referred to as the inducer skin graft, expressed both the target antigen and putative Th cell determinants. By using this experimental system, we found that H-2^b mice contained sufficient numbers of anti-bm6 Tk cells to rapidly reject bm6 mutant tailskin allografts, providing that the anti-bm6 Tk cells received T cell help. Thus, the fact that most H-2^b mice fail to reject bm6 tailskin allografts is due to a deficit in bm6-specific Th cells, confirming that Th cell function is the limiting, rate-determining step for rejection of K^b mutant skin allografts.

To more directly identify the T cell populations involved in skin allograft rejection, we developed an adoptive transfer model in which H-2^b nude mice were engrafted with allogeneic skin and then reconstituted with T cells or T cell subpopulations. In this experimental model the rejection of skin allografts was shown to depend upon and be mediated by the adoptively transferred T cells. Skin allografts expressing whole MHC disparities were rejected by negatively selected populations of either $CD4^+$ or $CD8^+$ T cells. In contrast, class I disparate skin allografts were only rejected by $CD8^+$ T cells, whereas class II disparate and minor H disparate skin allografts were only rejected by $CD4^+$ T cells. These results demonstrated that skin allograft rejection was not a function unique to one phenotypic subset of T cells, but could be mediated by either $CD4^+$ or $CD8^+$ T cells, depending upon the class of

histocompatibility antigens the graft expressed. The functional capabilities expressed in vitro by $CD4^+$ and $CD8^+$ T cells responding to a wide variety of different histocompatibility antigens was compared with their ability upon adoptive transfer to reject skin allografts expressing those histocompatibility antigens. These studies revealed that, without exception, skin allografts were only rejected by T cell populations that contained, by in vitro assessment, both lymphokine secreting Th cells and cytolytic Tk cells specific for the antigens of the allograft. Thus, skin allograft rejection responses appeared to require the activation of antigen-specific, lymphokine secreting Th cells and the subsequent generation of antigen-specific Tk cells. Further studies demonstrated that skin allograft rejection could result from T-T collaborations between lymphokine secreting Th cells and Tk cells that were of different CD4/8 phenotypes and of different antigen specificities.

These studies strongly supported the concept that in vivo rejection responses, once initiated by antigen-specific Th cells, are mediated by antigen-specific Tk cells. However, they did not directly demonstrate that antigen-specific Tk cells actually mediate the rejection process. Indeed, it was conceivable that allograft rejection is effected by lymphokines secreted by antigen-specific Th cells or DTH cells that either nonspecifically recruit inflammatory effector cells or induce intense vasospasm, causing nonspecific ischemic necrosis of the graft. To distinguish between antigen-specific and antigen-nonspecific effector mechanisms of allograft rejection, we constructed B6<->A/J allophenic mice which are genetic mosaics whose individual cells express either $H\text{-}2^b$ or $H\text{-}2^a$ determinants, but not both. The fur of these B6<->A/J allophenic mice consists of both black and white hairs, the black hair resulting from pigment donated by B6 ($H\text{-}2^b$) melanocytes and the white hair resulting from A/J ($H\text{-}2^a$) melanocytes that are genetically unable to synthesize pigment. Skin from these allophenic mice were grafted onto $H\text{-}2^b$ nude mice and rested for 1 month to permit the grafts to regrow their black and white hairs. Because nude mice are immunoincompetent, the experimental grafts were not rejected. The animals were then reconstituted with $H\text{-}2^b$ T cells responsive against the $H\text{-}2^a$ cellular elements of the allophenic grafts but tolerant to its $H\text{-}2^b$ elements. Within two weeks of the T cell transfer, the grafts were enveloped by an intense non-specific inflammatory response that caused all the hairs of the allophenic grafts to fall out. Nonetheless, the allophenic grafts survived the inflammation, and, most remarkably, regrew hair. The color of the regrown hair was overwhelmingly black, the color of the syngeneic $H\text{-}2^b$ parent, providing visual proof that the effector mechanism of skin allograft rejection destroys cells expressing allogeneic histocompatibility antigens but spares cells that do not. Thus, the effector mechanism of skin allograft rejection is MHC-specific and, therefore, mediated by a Tk cell that assesses individual cells in the dermis of the graft for expression of foreign histocompatibility antigens.

Publications:

Rosenberg, AS, Mizuochi T, Sharrow SO, Singer A. Phenotype, specificity, and function of T cell subsets and T cell interactions involved in skin allograft rejection. J Exp Med 1987;165:1296-1315.

Singer A, Munitz TI, Golding H, Rosenberg AS, Mizuochi T. Recognition requirements for the activation, differentiation, and function of T helper cells specific for class I MHC alloantigens. Immunol Rev 1987;98:143-170.

Rosenberg AS, Mizuochi T, Singer A. Evidence for involvement of dual function T cells in rejection of MHC class I disparate skin grafts. Assessment of MHC class I alloantigens as in vivo helper determinants. J Exp Med 1988;in press.

Rosenberg AS, Mizuochi T, Singer A. Cellular interactions resulting in skin allograft rejection. Annals N.Y. Acad Sci 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09276-04 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 05124-03 I

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

Expression and Function of Porcine Class I MHC Genes in Transgenic Mice

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

PI:	D. Singer	Senior Investigator	EIB, NCI
Others:	R. Ehrlich	Visiting Fellow	EIB, NCI
	W. Frels	Agricultural Research Service	USDA

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.50	1.25	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.)*

Porcine class I major histocompatibility genes have been introduced into the genome of C57BL/10 mice. To date, four transgenic animals containing distinct class I genes have been generated, and demonstrated to transmit the transgene to progeny. One transgenic line expresses SLA antigen on its cell surface and rejects skin of normal C57BL/10 mice, indicating that the foreign SLA antigen is recognized as a functional transplantation antigen. Expression of this SLA gene, PD1, parallels that observed in the swine, indicating that its expression is regulated. In vivo treatment of the transgenic with α,β-interferon increases PD1 expression in a number of tissues. This increase parallels that observed for the endogenous transplantation antigen, K^b, but differs markedly from the differentiation antigen, Qa-2.

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

Project Description

Major Findings:

Expression of individual class I genes is actively regulated: multiple genes within the family show similar patterns and responses, whereas other class I antigens, such as Qa and TL in the mouse, have been classified as differentiation antigens because of their limited tissue distribution. In order to further define the regulation of these genes, we are constructing transgenic mice, containing individual class I MHC genes from the swine. The swine genome contains seven class I MHC genes. To date, we have generated transgenic animals for three of these genes. All of these have been demonstrated to transmit the transgene and are currently being bred in order to establish lines. One of these, containing the gene PD1, has been sufficiently established to allow studies on the expression of the gene.

Analysis of RNA from swine tissues revealed that PD1 is expressed at highest levels in lymphoid tissues, and at lower but varying levels in a variety of other tissues, including thymus. Within the lymphoid compartment, PD1 expression is differentially regulated, as evidenced by the finding that PD1 is preferentially expressed in B cells, relative to T cells. Analysis of RNA from transgenic mouse tissues reveals that the pattern of PD1 expression, both qualitative and quantitative, parallels that observed in situ. Thus, regulatory elements necessary for the normal expression of PD1 are contained within the 9 kb pig DNA segment which was introduced into the transgenic mouse. Furthermore, trans acting factors involved in the regulation of class I MHC gene expression have been sufficiently conserved in evolution to allow them to recognize swine DNA elements. The PD1 transcripts present in the transgenic animal are functional, as evidenced by the fact that SLA antigen is observed on splenocytes, lymph node cells, and PBL. Taken together with the finding that the pattern of PD1 expression is indistinguishable from the H-$2K^b$ antigen, but distinct from Qa-2, these data indicate that PD1 encodes a swine classical transplantation antigen. Furthermore, skin grafts of transgenic donors onto normal mice are rejected, indicating that the SLA antigen can trigger graft rejection.

Since PD1 is interferon-responsive in transfected mouse L cells, its response to interferon in the transgenic animal was examined. Interferon induced PD1 expression in a variety of tissues, with the largest effects observed in the brain and heart; lower effects were observed in other tissues, with the exception of the kidney where there was no response. Within the lymphoid compartment, PD1 was induced to the same extent in both B and T cells. From these data we conclude that the DNA sequence elements necessary for in vivo interferon response are present within the transgene and that tissue specific elements determine the extent of the response. Using cell surface analysis of endogenous class I genes on spleen and thymus, it was demonstrated that H-$2K^b$, but not Qa-2, responded to interferon like PD1. Qa-2 expression in B cells was enhanced by interferon, whereas it was unaffected in T cells and thymocytes. From this we conclude: 1) a gene (i.e. Qa-2) can respond differently to the same immunomodulator in different cells and 2)

different genes in the same cell can respond differently to the same immunomodulator.

Because the class I MHC genes are members of a multigene family, in vivo DNAse I studies have not been possible for any species. Initial studies in pig spleen suggested that hypersensitive sites exist, as evidenced by the appearance of DNase I specific bands, but these bands could not be uniquely associated with a single gene. Transfected mouse L cells, in which PD1 expression is regulated, contain only a single copy of PD1, making it possible to examine its chromatin structure. We observed that the PD1 coding sequences were relatively nuclease sensitive, whereas the non-transcribed flanking sequences were relatively resistant. However, no DNase I hypersensitive sites were found within the pig DNA segment and no correlation with methylation was observed. Based on the observation that PD1 expression in the transgenic mouse is regulated and that its pattern of expression parallels that in situ, we turned to this model to test for DNase I hypersensitive sites within a class I gene in vivo. We reasoned that the presence of a site might identify the location of a trans acting factor, whereas the absence of a hypersensitive site would argue that it is not required for regulated expression. Analysis of nuclei from both spleen and thymus revealed the presence of a single, identical DNase I hypersensitive site in both tissues. As in vivo treatment of the transgenics with interferon did not alter the DNase I pattern of either tissue, this site appears to be constitutive and tissue non-specific. The site maps to a region 170 and 210 bp upstream of transcriptional initiation which is precisely the region where in vitro DNase I footprinting has mapped a binding site for an H-$2K^b$ trans acting factor (KbF1, H2TF1). Biological effects associated with this binding remain unclear since NFKb, an immunoglobulin binding factor, also recognizes this site. This region overlaps with the interferon responsive element, and has been reported to have enhancer activity in the H-$2K^b$ gene. Whether this site is actually associated with tissue specificity remains to be tested; however, if so, it is not by altering the site but rather the bound factors. Preliminary evidence suggests that the same binding factors can be isolated from cells of different lineages, indicating perhaps that this site is involved with basal levels of expression and not quantitative regulation.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09277-03 E

PERIOD COVERED
October 1, 1987 to September 30, 1988 formerly Z01 CB 09200-02 I

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of B Lymphocyte Subpopulations and Membrane Molecules

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

PI:	G. Laszlo	Visiting Fellow	EIB, NCI
Other:	H. B. Dickler	Senior Investigator	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

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 space provided.)*

The goal of this project is to identify and characterize B lymphocyte subpopulations and membrane molecules. Monoclonal antibody F1-10 detects a determinant (termed Ly-39.1) preferentially expressed on activated B lymphocytes. Expression peaks 60 hours after activation using LPS. The Ly-39.1 determinant is also expressed on splenic B lymphocytes and a subpopulation of bone marrow cells but at much lower levels and is absent from thymocytes, splenic T lymphocytes, and activated T lymphocytes. Mapping studies using BXD recombinant inbred mice indicate a gene controlling expression of the Ly-39.1 determinant is located on chromosome 17 between hbap-4 and acry-1. These results together with a unique strain distribution pattern suggest that Ly-39.1 is detecting a previously undescribed determinant selectively expressed on activated B lymphocytes which may be a receptor for lymphokines. A new hybridoma screening method has been developed using particle concentration fluorescence which is capable of detecting antibodies binding to molecules which are expressed at very low levels (100-300 molecules per cell). Studies of B lymphocyte subpopulations indicate that large "activated" B lymphocytes obtained directly from mice or B lymphoblasts induced in vitro with $F(ab')_2$ anti-mu significantly augment the responses of small "resting" B lymphocytes to $F(ab')_2$ anti-mu and lymphokines. Proliferation was augmented 2-4 fold while antibody production was augmented 4-5 fold. This effect was specific for "activated" B lymphocytes in that other cell types did not have this effect. Kinetic experiments revealed that the augmenting signal was effective after stimulation via antigen receptors but prior to the effects of lymphokines. The augmenting effect does not appear to be genetically restricted. Investigation of the nature of the signal revealed that neither supernatants nor plasma membranes from activated B cells alone augmented responses but both together did. These studies suggest that interactions between B lymphocytes are important in regulating humoral immune responses.

Project Description

Major Findings: A rat IgG2b,k mAb has been obtained which binds selectively to LPS stimulated B lymphoblasts. A majority of normal spleen B cells and 10-20% of bone marrow cells are also weakly positive (10-20 fold less). Thymocytes, normal T cells from spleen, and Concanavalin A stimulated T lymphoblasts are negative. Expression of the Fl-10 antigen peaks 60 hrs after stimulation with LPS and the kinetics of expression differ from those of sIgM, sIgD, Fcγ receptors and Ia antigens.

Monoclonal anti-FcγR and IgG anti-MHC class II antibodies inhibit binding of Fl-10, while IgG anti-class I and IgM anti-class II antibodies do not. However, FcγR and Fl-10 expression are discordant on certain tumor cell lines as well as in the kinetics of expression after LPS stimulation. Thus, these 2 molecules are probably not identical but may be associated.

Typing of LPS blasts from various mouse strains for Fl-10 expression reveal that DBA/2, DBA/1, AKR/J, NZB, MRL, and BXSB are intermediate or high expressors while C55BL/10, A/J, Balb/c, and C57L are low or negative for expression. This is a unique strain distribution pattern.

Mapping studies using BxD recombinant inbred mice reveals that a gene controlling expression of the Fl-10 determinant is located on chromosome 17 between hbaps-4 (3 mismatches in 22 strains) and acry-1 (3 mismatches in 20 strains).

It was concluded from the tissue distribution, kinetics of expression, strain distribution, and mapping studies that Fl-10 detects a previously undescribed B lymphocyte membrane molecule. The data are consistent with the possibility that this molecule is a lymphokine receptor.

Many molecules of importance on the B lymphocyte surface may be expressed at very low levels. Thus, it is important to develop a method which detects monoclonal antibodies specific for such molecules. We have recently successfully adapted particle concentration fluorescence immunoassay for use as a screening procedure. For this assay, lymphocyte aliquots are first incubated with supernatants from the clones to be screened (or known rat antibodies as controls) followed by FITC-mouse anti-rat Ig. The labeled cell samples are then loaded into specially designed plates which have 96 wells each of which has a 0.2 filter bottom. Protein coated 3.4 polystyrene beads are added to allow efficient filtration, the cells are filtered onto the bottom of the wells by application of vacuum, and total cell bound fluorescence in each well rapidly determined by front-surface fluorometry (i.e. fluorometry of the surface of the filter). Using rat monoclonal antibodies specific for known B lymphocyte surface molecules, we have been able to detect as few as 10^7 (and reproducibly $3x10^7$) molecules among 10^5 cells so that theoretically we can identify monoclonal antibodies which are specific for molecules whose expression is as low as 100-300 molecules per cell.

Purified (96% sIg positive, 0.1% T cells, 0.1% macrophages) small (120 ^{3} diam) dense (>1.087) normal B lymphocytes are poor responders to a variety of polyclonal stimulators including LPS and F(ab')$_2$ anti-mu with or without lymphokines.

Taken together with the fact that these cells have low background proliferation and antibody production, they are considered "resting" B cells. In contrast, purified (95% sIg positive, 0.5% T cells, 0.5% macrophages) large (170 μ^3 diam) less dense (1.075-1.081) normal B cells have higher background proliferation and antibody production, are brisk responders to the above stimuli and are considered "activated". Addition of the "activated" B cells (irradiated with 4000 R to prevent responses) to cultures of "resting" B cells stimulated with $F(ab')_2$ anti-mu and lymphokines significantly augmented the responses of the latter cells (2-4 fold for proliferation and 4-5 fold for PFC responses. Using 50,000 resting B cells, titrations revealed that 10,000 irradiated activated B cells were sufficient to maximally augment responses. Additionally, $F(ab')_2$ anti-mu induced B lymphoblasts demonstrated similar augmenting capacity. This activity was not found in other cell types including resting B lymphocytes, thymocytes, concanavalin A induced lymphoblasts or macrophages.

The augmenting activity of activated B lymphocytes appeared to be unique. Thus, increasing the amount of T lymphocyte-derived lymphokines would not substitute for this activity. Moreover, a series of time course and delayed addition experiments revealed that the activated B cells provided augumentation if added simultaneously or 24 hr after but not 24 hours before anti-mu stimulation, while they provided augmentation if added 24 hrs before or simultaneously with T lymphocyte derived lymphokines but not 24 hrs after. Thus the sequence of signals appears to be: anti-mu → activated B lymphocytes → lymphokines.

No evidence for genetic restriction of the functional interaction between "activated" and "resting" B cells was obtained. Thus, mixing experiments using DBA/2 and B10 B cells revealed equal augmuntation in syngeneic and allogeneic mixes. Similarly, (B6D2)F1 resting B cells were augmented equally by activated B cells from B6, DBA/2, $(B6D2)F_1$, or mixtures of B6 and DBA/2. Finally, anti-Ia antibody did not block augmentation.

Supernatants from activated B cells were prepared as well as plasma membranes from these cells and both were tested for augmenting activity. Neither the supernatant nor the membranes alone provided augmentation. However, a mixture of both did reconstitute the augmenting activity, while activated B cell supernatant plus thymocytes or thymocyte plasma membranes did not. Thus, the augmenting activity appears to have two components, one soluble and one membrane bound.

Publications:

Uher F, Dickler HB. Interactions between B lymphocyte subpopulations. Augmentation of the responses of resting B lymphocytes by activated B lymphocytes. J Immunol 140:1442-1447, 1988.

Park E, Principato MA, Lamers MC, Dickler HB. Ly-39: a new lymphocyte alloantigen defined with a monoclonal antibody. Immunogenetics, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09279-03 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 09203-02 I

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

Isolation and Characterization of a Novel H-2 Class I Gene

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

PI:	D. Singer	Senior Investigator	IB, NCI
Others:	S. Rudikoff	Senior Investigator	LG, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	1.75	

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.)*

A new sub-family of H-2 class I genes has been identified in the genome of the C57BL/10 mouse. The family consists of at least two genes which have not been previously identified. One of these, Mbl, has now been extensively characterized. Using a series of recombinant strains of mice, and taking advantage of restriction enzyme polymorphisms, it has been possible to map the Mbl gene to the right of the Qa locus. DNA sequence analysis of Mbl demonstrates that the exon organization of this gene resembles that of other class I genes. Furthermore, it is capable of encoding a transmembrane protein with a structure similar to other class I molecules. However, the level of DNA sequence homology of Mbl to other H-2 genes is no greater than to either human, pig, or rabbit. Furthermore, the over-all organization of Mbl is more similar to man and pig than to mouse. Whereas all previously reported H-2 class I genes have third introns of 1.2-2 kb, Mbl has an intron of 600 bp, similar to those of human and porcine class I genes. Taken together, these data suggest that Mbl may represent a direct descendant of a primordial class I gene, which antedates speciation. In support of this conclusion is the observation that a variety of wild mice, representing millions of years of evolutionary divergence, contain Mbl in their genomes. Although other members of the Mbl family appear to be expressed, no direct evidence for Mbl expression has been obtained, despite the fact that it is a structurally competent gene.

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

Project Description

Major Findings: The ability to isolate and characterize MHC genes makes possible studies on the evolution of this multigene family, as well as affords new approaches to identifying functionally important proteins. Many of the class I MHC genes of the mouse have been previously identified, and characterized as transplantation antigen genes or closely related differentiation antigen genes. However, there appears to be at least one sub-family of class I genes which is highly divergent from the rest. Since the existence of such a gene will change the possible models of the evolution of the family, we have conducted a careful study of the structure of this divergent gene, Mbl. In addition, it was of interest to examine the ability of this gene to be expressed. The product of such a divergent gene might be expected to have novel functions.

A genomic phage clone containing Mbl was isolated by low stringency hybridization with a human Class I cDNA probe. Following restriction enzyme mapping to localize the gene within the clone, DNA sequence analysis of the gene was performed. This analysis revealed that Mbl consists of five exons, encoding a leader, three extracytoplasmic domains, and a transmemebrane domain and intracytoplasmic anchor. Unlike other class I genes, no exons 6, 7, or 8 were found. All exons contain open reading frames and conserved splice sites. The gene terminates in a poly A addition site. At the 5' end, canonical promoter elements are found. The DNA sequence homology of Mbl to other class I genes revealed a surprisingly low homology to H-2 genes. Comparison with K, D, Qa, or Tl showed Mbl to be equally related to all of the sub-families, with an average homology of about 60% in the exons and about 30% in the introns. Comparison with class I genes from pig, human or rabbit showed that Mbl was no less related to these genes than to the murine genes. Analysis of the exon/intron organization of Mbl gave rise to the even more surprising finding that Mbl is more like non-murine genes than other murine genes. Thus, whereas all of the previously characterized murine genes have undergone a large insertion in the third intron, this has not occurred in either Mbl nor class I genes from other species. The converse situation obtains in intron 2 where Mbl, like other non-murine genes, is longer than other murine class I genes. These observations lead to the speculation that Mbl has evolved from an ancestral gene which antedates speciation and that Mbl has not participated in the various gene conversion events that have occurred within the rest of the class I family. Supporting this notion is the observation that Mbl is found among wild mice, even those separated in evolution by up to 6 million years.

Using a series of recombinant mice, it hs been possible to demonstrate that Mbl maps to the right of the Qa locus. The precise location is unknown, since the appropriate recombinants are not available.

Although Mbl by DNA sequence analysis appears to have the capacity to be expressed, it has not been possible to demonstrate expression. Transfection of mouse L cells with Mbl does not result in specific transcripts. Analysis of a variety of tumor cell lines, normal tissue from adult and embryonic mice, or activated splenic T cells gives no evidence for the presence of an Mbl transcript. Thus, the function of the Mbl gene, if any, remains to be determined. It has been demonstrated that other members of the Mbl family are expressed in normal tissues, as well as in a variety of tumors. However,

attempts to isolate a cDNA clone representative of the family have not yet been successful; under conditions of high stringency, only classical class I cDNAs were found.

The major signifiance of the findings on Mbl are 1) the presence of a new subfamily of class I genes raises the possibility that there may be other, equally distantly related class I genes, which remain to be identified. Thus, the number of class I gens in the murine genome, already the highest among species examined, may be even higher; 2) the fact that Mbl appears to have evolved independently of the rest of the class I family raises interesting questions about the evolution of a multi-gene family; and 3) the observation that despite the extensive divergence between Mbl and the rest of the family, there have been no incapacitating mutations suggests that Mbl may be a functional gene.

Publications:

Singer D, Hare J, Golding H, Flaherty L, and Rudikoff S. Characterization of a new subfamily of class I genes in the H-2 complex of the mouse. Immunogenetics 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09281-03 E

PERIOD COVERED
October 1, 1987 to September 30, 1988 formerly Z01 CB 09205-02 I

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Receptor Mediated T 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	EIB, NCI
Others:	K. S. Hathcock	Chemist	EIB, NCI
	D. M. Segal	Senior Investigator	EIB, NCI
	M. Taplits	Medical Staff Fellow	EIB, NCI
	P. Bohjanan	Howard Hughes Fellow	EIB, NCI
	K. Kelly	Senior Investigator	EIB, NCI

COOPERATING UNITS *(if any)*
Food and Drug Administration

LAB/BRANCH
Experimental Immunology Branch

SECTION
Immunotherapy Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

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 standerd unreduced type. Do not exceed the space provided.)*

The mechanism of T cell activation has been studied employing both cloned T cell populations and naive heterogeneous T cell populations. Comparison has been made of T cell stimulation by antibodies directed to allotypic determinants on the T cell receptor, T cell activation by specific antigen, and T cell activation by non-receptor-mediated signaling. It has been demonstrated that both cloned and naive T cells can be triggered by the monoclonal antibody F23.1, directed toward determinants on the T cell receptor. Naive $Lyt2^+$ T cells were activated to proliferate in the presence of soluble F23.1, IL-2, and accessory cells. Under the same conditions, $L3T4^+$ naive T cells were unresponsive. These findings thus demonstrated a difference in the activation requirements of T cell subpopulations triggered through T cell receptor determinants. Specific "targeting" of the T cell receptor to accessory cell structures by heteroaggregates of F23.1 coupled to monoclonal anti-Ia antibody were, in contrast, capable of activating both $Lyt2^+$ and $L3T4^+$ T cell subpopulations. The nature of activation signals provided under these diverse conditions is currently under study.

The role of endogenous lymphokines in T cell activation has been demonstrated. The proliferative response of type 2 T helper clones is inhibited by antibody specific for the lymphokine IL4, which is produced by these clones. This demonstrates that IL4 acts as an autocrine growth factor for these T cells. In addition, mRNA levels corresponding to a number of activation genes are inhibited by the presence of anti-IL4 antibody during T cell activation. In contrast, the level of IL4 mRNA is actually increased in the presence of this antibody, suggesting that an endogenously produced IL4 may normally down regulate its own transcription.

Project Description

Major Findings:

1. Activation of Naive T Cells.

Naive splenic T cells were fractionated by treatment with anti Lyt2 or L3T4 plus complement. Unfractionated or fractionated T cell populations were then cultured in the presence of stimuli including F23.1 soluble antibody, sepharose-bound F23.1, anti Thy1.2 antibody, Con A, recombinant IL-2, or heteroaggregates of F23.1 linked to other monoclonal antibodies. Vigorous proliferation was induced in unfractionated or $Lyt2^+$ T cells by soluble F23.1 in the presence of IL-2 and accessory cells. In contrast no significant proliferative responses were observed in $L3T4^+$ T cells. The same pattern was observed with sepharose-bound F23.1 antibody. In contrast, the Fab of F23.1 covalently linked to the Fab of anti-A^k A^k antibody was capable of activating $Lyt2^+$ or $L3T4^+$ T cells even in the absence of IL-2 but in the presence of A^k A^k-positive accessory cells. This activation mediated by heteroaggregate antibody required specific targeting by covalently linked antibodies, and was not due to the independent effects of F23.1 and anti Ia antibodies. Proliferation of naive T cells was also induced by antibodies to a component of the T3 complex, in the absence of exogenous lymphokine. Under these conditions, proliferation was substantially inhibited by antibodies to IL-2 or to IL-4, suggesting a possible role of endogenously produced lymphokines in the proliferative response of T cells to this stimulus. Activation of naive T cells was also assessed by the measurement of gene induction. Increased levels of myc and IL-2 receptor mRNA were induced in response to anti-T3, F23 + IL-2, or Con A.

2. Activation of Cloned T Cells.

Both $L3T4^+$ and $Lyt2^+$ cloned T cells were assayed for expression of the F23.1 allotypic determinants. Certain T cell clones in each category were positive. F23.1 expressing T cells could be triggered to proliferation in the presence of F23.1 soluble antibody and accessory cells. In these circumstances, there was no requirement for specific recognition of MHC determinants on accessory cells by responding T cell clones. Re-targeting by heteroconjugate antibodies also produced enhanced proliferative responses by T cell clones and was again specific for cross linking of T cell receptor structures to determinants on the surface of accessory cells. F23.1 antibody immobolized either on plastic tissue culture wells or on polystyrene beads was capable of activating cloned T cells in the absence of accessory cells or exogenous lymphokines. It was also demonstrated that, similar to previous findings for cytotoxic T cells, cloned helper T cells contain cytoplasmic granules which in turn are associated with a number of enzymatic activities. Release of cytoplasmic granules by cloned T cells was studied in response to a variety of stimuli. IL-2, specific antigen, anti-T3, or F23.1 were all capable of inducing proliferation in a number of helper cloned T cell populations. In contrast, IL-2 failed to induce any increased release of cytoplasmic granules, whereas each of the T cell receptor-mediated stimuli noted above was capable of inducing granule release.

3. Function of Endogenous Lymphokines in T Cell Activation.

Cloned T helper cells produce a number of now well characterized lymphokines and in addition are themselves responsive to a number of lymphokines. One class of T cell clones, termed type 2, produces IL4 and is induced to proliferate in response to exogenous IL4. The fact that IL4 acts as an autocrine growth factor for these cells was demonstrated. Proliferation and T cell activation were induced by immobilized antibodies specific for the T3 complex of the T cell receptor. Under these conditions, proliferation was blocked by the presence of antibody to IL4. Activation of other genes including the proto-oncogenes c-myc and c-myb were also inhibited by the presence of anti-IL4 antibody. T cell activation mediated through the T cell receptor complex is thus dependent upon an autocrine role of IL4. In contrast to these inhibitory influences, it was observed that the presence of anti-IL4 antibody resulted in a significant increase in steady state IL4 mRNA levels. These latter data suggest that the presence of endogenously produced IL4 normally down regulates the production of further IL4, and that it does so at the level of gene transcription or of message stabilization.

Proposed Course of Project: The consequences of T cell binding by anti receptor antibodies will be studied at a biochemical level. Influences of receptor binding on early gene activation, ion flux and IL-2 receptor expression will be assessed. In addition, the target structures involved in T cell interaction with cooperating B cells or accessory cells will be probed through the use of heteroaggregates between F23.1 and antibodies directed at a variety of cell surface determinants expressed on both T and non T cells. The role of endogenously produced lymphokines on T cell proliferation as well as on specific gene induction are now being evaluated.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09282-02 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 Formerly Z01 CB 09207-01 I

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

T Cell Immune Deficiency in Mice and Humans With Autoimmune Disease

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

PI:	G. M. Shearer	Senior Investigator	EIB, NCI
Other:	C. S. Vià	Medical Staff Fellow	EIB, NCI
	G. C. Tsokas	Senior Investigator	ARB, NIADDKD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

B

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

Spleen cells from Mrl-lpr mice gradually develop a loss of $CD4^+$ T helper cell function that is age-dependent which is concomitant with the appearance of anti-DNA antibodies and of suppressor cells that selectively suppress only $CD4^+$ T helper cell function. These suppressor cells were found to be T lymphocytes of the L3T4 phenotype. This observation contrasts with Mrl +/+ mice that exhibit none of the above during the same time period.

Twelve of fifteen human patients with SLE exhibit a similar selective loss of CD4 T helper cell function.

Project Description

Major Findings: Based on our findings that mice undergoing the autoimmune form of GVH (see #05088) exhibit a selective defect in $CD4^+$ T helper cell function, and generate suppressor cells that are selective for $CD4^+$ T helper cells, we have tested whether mouse strains that make anti-DNA antibodies naturally exhibit a selective defect in $CD4^+$ T helper cell function and generate suppressor cells that selectively suppress $CD4^+$ T helper cells. We found that spleens of two-month old Mrl-lpr mice exhibited normal T cell functions, whereas four- and six-month old Mrl-lpr mice exhibited both deficient $CD4^+$ T helper cell function and suppressor cell activity selective for $CD4^+$ T helper cells. Mrl +/+ mice of the same ages did not exhibit these defects. These results raise the possibility that anti-DNA antibody production activates this unusual suppressor cell population which is an attempt to down-regulate $CD4^+$ T helper cell function.

Recent studies indicate that other models of B cell hyperactivity including B cell activation by lipopolysaccharide or the natural B cell hyperactivity that occurs in aging SJL/J mice are associated with selective suppression of $CD4^+$ T helper cell function. Preliminary studies with PBL from patients with SLE also exhibited a loss in $CD4^+$ T cell function in 12 of 15 patients tested.

Publications:

Via CS, Shearer GM. T-cell interactions in autoimmunity: Insights from a murine model of graft-versus-host disease, Immunol. Today 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09283-02 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 Formerly Z01 CB 09208-01 I

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

Regulation of Human T Cell Responses by Adherent Cells

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

PI: G. M. Shearer Senior Investigator EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	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.)*

Depletion of human peripheral blood leukocytes (PBL) of Leu $M3^+$ cells by adherence to plastic and sephadex G10 results in the abrogation of proliferative (^3H) and cytotoxic T lymphocyte (CTL) responses to influenza A virus (FLU), but in an elevation of ^3H and CTL responses to HLA alloantigens (ALLO). This loss of the FLU T cell response was attributed to removal of antigen-presenting cells (APC), whereas the elevation of the ALLO response was shown to be due to a suppressor cell (or a suppressor inducer cell) that is contained in the Leu $M3^+$ adherent cell population. Suppressor activity was inactivated by culturing either unfractionated PBL or adherent cells with viruses, including influenza A, measles and mumps viruses.

Project Description

Major Findings: PBL from HIV^+ donors were studied for their ability to generate proliferative and CTL responses to HLA alloantigens. PBL from approximately 70% of HIV^- donors generated weak or only marginal CTK responses to ALLO, while they generated strong CTL responses to FLU in the same experiments. PBL from the other 30% of donors generated strong ALLO CTL responses. The phenomenon was donor-selective in that those donors who were poor CTL responders to ALLO remained poor responders for more than two years, and poor responsiveness was observed irrespective of the source of ALLO stimulators and irrespective of whether a single donor or a pool of ALLO stimulators was used. Proliferative responses to ALLO were also reduced, but not as much as CTL responses. The ALLO CTL responses of these weak responders was elevated to the levels observed for FLU-specific CTL in the same donors and to the levels observed for ALLO responses in many HIV^+ donors and AIDS patients, but procedures that removed macrophages from the responder population. Addition of irradiated, autologous unfractionated PBL or irradiated, autologous macrophage-enriched PBL suppressed the level of CTL activity to that of unfractionated PBL.

The fact that this suppression appeared to affect ALLO CTL, but not FLU CTL activity prompted us to question whether influenza virus in some way abrogated suppressor cell activity. We found that suppressor cell activity was abolished by incubation of PBL with influenza virus.

Publications:

Bernstein DC, Shearer GM, Suppression of human cytotoxic T lymphocyte responses by adherent peripheral blood leukocytes. Ann. N.Y. Acad. Sci., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09285-02 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 09210-01 I

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

Induction of Class I MHC Gene Expression by Ethanol

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

PI:	Dinah Singer	Senior Investigator	EIB, NCI
Others:	M. Kolber	Medical Staff Fellow	EIB, NCI
	M. Hinners	Chemist	EIB, NCI
	R. Wall	Guest Researcher	GWU Hospital

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.27	0.27	

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.)*

Ethanol enhances expression of cell surface class I major histocompatibility complex (MHC) antigens in a variety of cell lines; up to an eightfold increase is observed in an embryonic cell line. Increased cell surface expression, following alcohol treatment, occurs with a concomitant increase in steady-state RNA levels. This effect is promoter dependent and restricted. The effective ethanol concentration (1%) is physiologically attainable. Measurement of class I MHC antigen on peripheral blood lymphocytes in acutely ethanol intoxicated patients showed a highly significant increase over controls.

Project Description

Major Findings:

Treatment of a variety of cell lines, including thymomas, fibroblasts, and embryonic cells, with ethanol increases the level of class I MHC gene expression on their cell surface. In cell culture, the effect is not restricted to class I MHC antigens; levels of transfected class II gene products also increased. It has been demonstrated that intracellular protein synthesis increases, as do steady state RNA levels.

These observations led to the speculation that in vivo, ethanol may also induce increased levels of class I MHC gene expression, which might lead to auto-immune components in alcohol-related diseases. A group of acutely intoxicated patients was tested for PBL class I MHC levels. It was found that there is a significant increase in the level of cell surface expression of class I antigens in the patient population, relative to normal controls. In contrast to the observations in cell culture, no increases in class II levels were noted.

To pursue the in vivo effects of ethanol, studies have been initiated in murine models. It has been found that ethanol treatment of mice in an environmental chamber does not elevate class I levels. Assessment of a spontaneously drinking mouse is currently underway.

Publications:

Parent L, Ehrlich R, Matis L, and Singer D. Ethanol: An enhancer of major histocompatibility complex antigen expression. FASEB J 1987;1:469-473.

Singer D, and Kolber M. FASEB J 1988;2:2324. (Letter to the Editor)

Kolber M, Walls R, Hinners M, and Singer D. Evidence of increased class I MHC expression on human peripheral blood lymphocytes during acute ethanol intoxication. Alcoholism: Clinical and Experimental Research 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09286-02 E

PERIOD COVERED

October 1, 1987 to September 30, 1988 formerly Z01 CB 09211-01 I

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

Cell Type Specific Regulation of the T Cell Receptor β Chain

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

P.I.:	K. Kelly	Senior Investigator	EIB, NCI
Others:	M. Kearns	Guest Researcher	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.20	1.20	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 genetic regulatory mechanisms that govern tissue specific expression of the T cell receptor (TCR) β chain have been investigated utilizing an in vitro model of cell type specificity. Both transient and permanent transfection systems have been used to assay the transcription of a genomic TCR β chain gene in T cells, fibroblasts, and a variety of hematopoietic tumor cells. The nature of the vector containing the genomic β chain gene as well as the relative amount of 5′ untranslated sequence contained within the genomic clone influence the degree of tissue specificity observed. TCR β chain genes containing 5 kb of 5′ UT sequence within a vector which contains polyoma enhancer and replication origin sequences display no tissue specificity of expression. By contrast, the same β chain gene within a pBR vector containing no eucaryotic regulatory sequences shows preferential expression as expected in T cells as compared to fibroblasts. Thus, unlike immunoglobulin genes, tissue specificity of expression as determined by resident regulatory sequences within a genomic TCR β chain gene are dominantly influenced by external eucaryotic regulatory sequences. In addition, TCR β chain genes containing 1.5 kb of 5′ UT sequence within a pBR vector display no preferential tissue expression. These results imply that a tissue-specific negative regulatory element exists within the deleted 3.5 kb of upstream sequence.

GPO 914-916

Project Description

Major Findings:

Identification of the genetic regulatory mechanisms that govern tissue specific expression of the T cell receptor (TCR) β chain in the mouse has been investigated utilizing an in vitro model of cell type specificity. A rearranged genomic clone for the TCR β chain was transfected into various lymphoid cells and fibroblasts to determine whether or not the exogenously introduced β chain is regulated in a tissue specific manner. This issue was addressed utilizing the entire gene with either 5 kb or 1.5 kb of 5' untranslated (UT) sequence in both permanent and transient transfections.

Genes were introduced into T cells, B cells, monocytes and fibroblasts via DEAE mediated gene transfer in hypotonic media. Transfection efficiencies were normalized by determining the mRNA levels of a truncated histone gene contained within the plasmid vector as a tissue nonspecific control. This vector (Py5) also contains a polyoma origin of replication to increase the copy number of linked genes and has been used to define the genetic regulatory elements for the immunoglobulin heavy chain gene. The expression of transfected genes was subsequently analyzed by S1 nuclease protection. This allows identification of the start site of transcription and whether or not correctly initiated sequences occur in non-T and non-lymphoid cells. Unlike immunoglobulin genes, expression of a rearranged Vβ1 Jβ2Cβ1 gene occurs in non-lymphoid cells. The elimination of 3.5 kb of 5' untranslated sequence improves the expression of the transfected gene in T cells four fold over that seen with the entire 5 kb of 5' upstream sequences. This implies that a negative regulatory element may be operative within this region.

Stable transfectants which permanently express the TCR β chain gene with either 5 or 1.5 kb of 5' UT sequence were established in T cells and fibroblasts using the Py5 vector, confirming the results seen in transient transfections. Preferential expression in T cells can occur, however, when the β chain is permanently transfected in a pBR vector which does not contain any eucaryotic enhancer and/or replication associated sequences. These results indicate that the polyoma origin of replication can cause inappropriate tissue expression of linked genes. Without this element, the TCR β chain is not expressed in fibroblasts when 5 kb of 5' UT sequence is included. If, however, 3.5 kb of 5' UT sequence is eliminated, the TCR β chain gene is once again expressed in permanently transfected fibroblasts. These results were confirmed in several clones with different integration sites. This implies that a negative regulatory element exists within the deleted 3.5 kb of upstream sequence. This finding has significant implications with regard to the tissue specific regulation of this gene. Perhaps the suppression of V region expression in fibroblasts effects gene rearrangement by preventing formation of an open chromatin structure in this region in non-T cells. Future experiments to address this issue involve fine structure analyses to identify the location of this element. Footprinting and/or gel shift analyses may also be used in the identification of binding sites for proteins within this region.

DNA sequencing of the Vβ1 leader and an additional 400 bp 5′ was done in order to identify promoter elements and allow the identification of putative regulatory sites. Conserved sequences have been shown to be functionally important within defined enhancer and/or regulatory regions of many genes. Identification of conserved sequences by comparison to other published β chain sequences would allow a preliminary focus of interesting regions for further study. A putative regulatory hexamer, CTTTGT, that is conserved in several human and murine Vβ genes is approximately 250 bp 5′ to the mRNA cap site. The functional significance, if any, of this remains to be analyzed by appropriate deletion studies and introduction of numerous repeat copies of this hexamer 5′ to the CAT gene with the TCR promoter and heterologous promoters. Transfection of these constructs into various cell types would indicate whether or not this sequence has functional significance in the regulation of β chain expression.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09287-01 E

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Marrow Graft Failure in Allogeneic Bone Marrow Transplantation

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

PI:	Ronald E. Gress	Senior Investigator	EIB, NCI
Others:	Hirotoshi Nakamura	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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,D

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

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL) may play a significant role in mediating allogeneic marrow graft rejection. In a murine model system, CTL were cloned from the spleens of sublethally irradiated animals which had rejected MHC disparate marrow grafts. It was found that cloned CTL were sufficient to effect rejection of T cell depleted allogeneic marrow in lethally irradiated animals. The rejection of marrow grafts by CTL was specific for the MHC gene products expressed by the marrow cells and correlated with the cytotoxic specificity of the individual clones. Although CTL can therefore reject marrow grafts, the administration of anti-CD3 monoclonal antibody in vivo has to date not enhanced engraftment of T cell depleted marrow in sublethally irradiated murine hosts. Cloned xenospecific human CTL were also directly evaluated for their ability to mediate tissue damage in vivo and were found to mediate tissue injury with specificity for MHC antigens of the murine host. The fine specificity of xenoantigen recognition by human T cells suggests that in transplantation of xenogeneic tissue, as in transplantation of MHC mismatched allogeneic tissue, the particular tissue antigens, and therefore tissue typing, may be a relevant consideration. The generation of human CTL has been characterized and certain essential cellular interactions in that generation have been identified. In addition, the mechanisms for the blocking effects of monoclonal antibodies specific for the CD2 (sheep red blood cell receptor), CD8, and CD18 (LFA-1) molecules expressed on the surface of human CTL have been investigated. Results indicate that the inhibition by anti-CD2 antibody did not occur in the absence of target cells, consistent with the interpretation that this antibody exerts its blocking effect by interfering with a CD2-ligand interaction. However, results with anti-CD8 and anti-CD18 monoclonal antibodies indicated that inhibition by these agents could not be explained by simple disruption of a T cell receptor-target cell ligand interaction.

Project Description

Major Findings:

The purpose of these studies was to directly assess the ability of murine CTL from irradiated hosts to reject allogeneic marrow grafts. First, the maximum dose of radiation which still allowed an effective rejection response by the host was determined. By an ^{125}IUDR uptake assay of splenic marrow cell proliferation, this dose was found to be 650-750 cGy. Lymphocytes were then isolated and cloned from spleens of B6(H-2^b) mice seven days after they had received 650 cGy radiation and inoculation with either BDF1 (H-$2^{b/d}$) or B6C3F1 (H-$2^{b/k}$) marrow which had been depleted of T cells by rabbit anti-mouse brain antiserum plus complement. The clones which were recovered were Thy 1+, LyT 2+, L3T4- with cytolytic specificity for the allogeneic MHC-encoded determinants of the marrow donor. No NK activity could be identified. It was found that these clones suppressed MHC mismatched marrow graft proliferation (measured by ^{125}IUdR uptake) when adoptively transferred into a 1025 cGy lethally irradiated B6 host if and only if the grafted marrow cells expressed MHC determinants for which the individual clone had cytotoxic specificity. Two primary questions were then addressed. The first was the effect of these CTL cells on long term engraftment and the second was the mechanism by which they exerted their effect. Results demonstrated that (1) a cloned CTL population is sufficient to reject an allogeneic marrow graft, and (2) the mechanism by which these marrow grafts were rejected was specific for MHC gene products expressed by the donor marrow corresponding to the cytotoxic specificity of the clone.

With the demonstration that CTL are sufficient to effect marrow graft rejection, a study was undertaken to evaluate the possible beneficial effects of anti-CD3 treatment on marrow engraftment in a mouse model (parallel to anti-CD3 monoclonal antibody treatment which is currently available for clinical use in allogeneic organ transplantation). The antibody used is specific for the ϵ chain of the murine CD3-T cell receptor complex, can suppress skin graft rejection, and can cause both short term and long term in vivo T cell dysfunction. T cell immunosuppression is pronounced at one week after administration of the antibody. It was found, however, that in vivo treatment with anti-CD3 administered seven days before infusion of bone marrow did not enhance engraftment of allogeneic marrow in sublethally irradiated hosts. Therefore, immunosuppression provided by treatment with anti-CD3 monoclonal antibody was not sufficient to prevent rejection of the marrow graft. More recent studies have demonstrated that administration of anti-CD3 to mice results in T cell activation within hours of administration, manifest by increased IL-2 receptor expression and by enhanced proliferation of spleen cells from treated animals to exogenous IL-2 in vitro. This activation also results in secretion of colony stimulating factors (CSF) detectable in the serum and produces extramedullary hematopoiesis in the spleen. These results indicate that it may be beneficial in terms of marrow stimulation and engraftment to time the injection of allogeneic marrow so that it is present during this period of T cell activation in order to provide the possible benefit of factors which stimulate hematopoiesis.

Information about human CTL generation, specificity, and in vivo function is not as extensive as in the case of murine CTL. These studies of human anti-mouse CTL responses indicate that both CD4+ and CD8+ helper pathways function in the generation of human CTL responses and that there exists a dependence on the presence of human antigen presenting cells. Additional experiments were carried out to characterize the human effector CTL subsequent to its generation. CTL lines and clones were established from a secondary mixed lymphocyte response in which the responder cells were human and the stimulators were H-2^b murine splenocytes. These human xenoreactive CTL were able to distinguish wild type K^b molecules from those altered in their amino acid sequence by in vitro mutagenesis. Such amino acid changes were confined to a single domain and so demonstrated a fine specificity of recognition by human CTL comparable to the specificity of recognition of K^b determinants by alloreactive murine CTL. This fine specificity of antigen recognition by human CTL across species predicts that the recognition by host CTL of MHC encoded antigens expressed by a xenograft would be expected to be comparable in specificity to the recognition of MHC antigens expressed by an MHC mismatched allograft.

These xenoreactive CTL populations were also used to evaluate whether isolated human CTL can mediate tissue injury in vivo. Human CTL were injected intradermally into murine hosts which either did or did not express the MHC determinants for which the cytotoxic populations were specific. In all instances in which an anti-H-2^b human CTL line or clone was injected into an H-2^b host, anatomical and histological changes developed. Anatomical lesions were first detected at 48 hours with progression to eschar formation by 96 hours. Histologically, exocytosis and toxic epidermal necrolysis was present at 48 hours with lymphocytic infiltration into the epidermis.

To investigate the blocking of CTL function by monoclonal antibodies, we have carried out blocking studies with CD2, CD3, CD8, and CD18 specific monoclonal antibodies in vitro. Each of these antibodies demonstrated some ability to inhibit the generation or function of CTL. The CD2, CD8, and CD18 molecules expressed on T cells are generally felt to function through the binding of ligands expressed by target cells. These ligands are LFA-3, Class I MHC determinants, and ICAM-1, respectively. The ability of monoclonal antibodies specific for CD2, CD8, and CD18 to block CTL function is therefore felt to be through a disruption of the normal interactions of these molecules with ligands on the stimulator/target cell. To investigate whether disruption of receptor-ligand interaction is in fact the mechanism of action of blocking by these agents, an assay of CTL function was developed which is independent of target cells. Two antibodies blocked in this system, anti-CD8 and anti-CD18. The ability of anti-CD8 to block, together with results obtained in blocking the killing of Class I negative targets, makes it unlikely that the mechanism of inhibition depends on a disruption of a CD8-target cell Class I interaction. In addition, a CD8+, Class II specific CTL clone, which was also blocked in the target cell-independent assay system, was not blocked by anti-CD8 monoclonal antibody in its lysis of target cells expressing the Class II antigen for which it is specific, suggesting that CD8 specific antibody does not inhibit CTL function by generating an absolute off signal. There was profound inhibition by anti-LFA-1 (CD18). Secretion induced by ionomycin +/- PMA was not inhibited, thereby demonstrating that the antibody did not act at

the level of secretion. These experiments strongly suggest that anti-LFA-1 inhibition, which has been utilized to a limited extent clinically in allogeneic bone marrow transplantation, may involve more than a disruption of the CD18 - ICAM-1 pathway of adhesion.

Publications:

Gress R, Moses R, Suzuki T, Lowman M, Sakamoto K, Pennington L, Sachs DH. Biparental bone marrow transplantation as a means of tolerance induction. Transplant Proc 1988;19:107.

Pennington L, Sakamoto K, Popitz-Bergez FA, Pescovitz MD, McDonough M, McVittie T, Gress RE, Sachs DH. Bone marrow transplantation in miniature swine. I. Development of the model. Transplantation 1988;45:21.

Popitz-Bergez F, Sakamoto K, Pennington L, Pescovitz MD, McVittie T, Gress R, Sachs D. Bone marrow transplantation in miniature swine. II. Effect of selective genetic differences on marrow engraftment. Transplantation 1988;45: 27.

Hirsch R, Bach J, Chatenoud L, Gress R, Sachs D, Bluestone J. Suppression of the humoral response to anti-CD3 mAb either by pretreatment with anti-CD4 mAb or with high dose anti-CD3 mAb. Transplant Proc 1988;in press.

Bluestone J, Ellenhorn J, Pluznick D, Gress R, Hirsch R. In vivo administration of anti-murine CD3-E mAb activates T cells. Transplant Proc 1988;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09288-01 E

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: T Cell Responses in Bone Marrow Transplantation

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

PI:	Ronald E. Gress	Senior Investigator	EIB, NCI
Others:	Robert Moses	Biotechnology Fellow	EIB, NCI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Experimental Immunology Branch

SECTION:

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

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,D

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

Concerns that a functionally effective T cell repertoire might never develop in the setting of MHC-mismatched marrow transplantation are based on the defective T cell responses in vivo observed in murine bone marrow chimeras constructed between MHC disparate strains. The incompetence is not due to inherent T cell defects, but rather to the restriction specificities of the T cells such that those MHC determinants recognized as self (host type) are not expressed by the antigen presenting cells which are of donor type. The T cell response to antigen is therefore defective. To evaluate whether T cell maturation is comparable in mouse and man, the development of T cell responses in a primate, the rhesus monkey, has been studied. The biology of T cell recovery has first been investigated in animals following T cell depleted autologous marrow transplantation. The time course of CD4+ T cell reconstitution and the time course of the development of in vivo T cell immunocompetence as defined by the ability to respond to an organ allograft correlated with the number of T cells infused in the marrow, and did not correlate with marrow cell dose or with time of hematopoietic reconstitution, raising the possibility that residual T cells in the infused T cell depleted marrow played a central role in the generation of susequent T cell populations. This would imply that the generation of T cells in marrow grafted primates may not be the same as in the mouse.

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

Project Description

Major Findings:

As a preliminary step to the study of T cell repertoire generation in MHC-mismatched BMT, we have studied the generation of T cell function in rhesus monkeys which have been hematologically reconstituted with either untreated or extensively T cell depleted autologous bone marrow. The methods for T cell depletion and the quantitation of residual (reinfused) T cells were patterned after methods developed for carrying out bone marrow transplantation in man. By phenotypic analysis, CD2+/CD8+ T cells recovered by 6-8 weeks post grafting. CD16+ NK cells and CD20+ B cells also recovered at 6-8 weeks. All animals receiving T cell depleted marrow recovered CD4+ cells at later time points. In the animal receiving marrow containing the fewest residual T cells (0.00014% by limiting dilution assay), CD4+ cells were less than 30% of the pretransplant value at ten months after transplant. The total number of T cells required to acutely reject a graft was determined on the basis of results in animals receiving marrow poorly depleted of T cells, and was 5 x 10^4 per kilogram recipient body weight. In vitro functional studies paralleled the phenotypic analysis. CTL activity, which can be mediated by CD8+ cells, was observed in vitro (with added IL-2) at six weeks post transplant; recovery of helper T cell function, predominately mediated by CD4+ cells, was found only at time points beyond four months. The ability to respond to allogeneic stimulation in vivo as assessed by the rejection of an organ allograft temporally paralleled the recovery of CD4+ helper cells, not the recovery of CD8+ cytotoxic cells. The critical level of recovery of peripheral CD4+ cells which correlated with allograft rejection was 25-50% of the pretransplant value. Approaches to evaluate T cell function by direct stimulation of CD3 were also developed and characterized.

The pattern and time course of reconstitution of T cell populations establishes a baseline for comparing T cell reconstitution in allogeneic donor/recipient combinations and in predicting the time required for successful treatment to reverse virally induced T cell deficiencies. Prolonged periods of depressed T cell number and function existed even in the absence of the allogeneic effects which might be expected in allogeneic BMT. The slow rate of recovery of CD4+ cells as compared to CD8+ cells may reflect distinct mechanisms in the generation of these two subsets. Also, even with very extensive T cell depletion, hematopoietic recovery occurred, indicating that hematopoiesis does not absolutely require many, if any, "hematopoietic T helper cells".

The length of time required for reconstitution of CD4+ cells and for recovery of organ allograft rejection varied inversely with the number of residual T cells in the infused marrow, not with stem cell function as assessed by the number of marrow cells infused and by rapidity of overall hematopoietic recovery. This result is consistent with the possibility that the residual T cells in the infused marrow play a central role in the generation of subsequent T cell populations in the recipient. This suggests that the generation of T cells in the primate marrow recipient may in fact be different than in the mouse where the T cell repertoire appears to develop

from marrow precursor cells which mature in the irradiated recipient and which consequently express an MHC restriction which is strongly influenced by the host environment. The possibility that reconstituting T cells in the primate following marrow transplantation are derived from mature donor T cells (with restriction specificity for donor MHC antigens) remaining in the marrow after depletion rather than from early precursors/stem cells is of central importance to considerations of MHC mismatched BMT in man.

Publications:

Moses RD, Orr KS, MacVittie TJ, Gress RE. Cardiac allograft survival across MHC barriers in the rhesus monkey following T lymphocyte-depleted bone marrow pransplantation I. T lymphocyte depletion studies. Transplantation 1988;in press.

Singer A, Munitz TI, Gress RE. Specificity of thymic selection and the role of self antigens. fTransplant. Proc 1987;19:107.

Kolber M, Quinones R, Gress R, Henkart P. Measurement of cytotoxicity by target cell release and retention of the fluorescent dye biscarboxyethyl-carboxyfluorescein (BCECF). J Immunol Methods 1988;108:255.

Calamonici O, Ang S, Quinones R, Henkart P, Heikkila R, Gress R, Felix C, Kirsch I, Longo D, Marti G, Seidman J, Neckers L. IL-2 dependent expansion of CD3+ large granular lymphocytes expressing T cell receptor gd: evidence for a functional receptor by anti-CD3 activation of cytolysis. J Immunol 1988;140:2527.

Calamonici O, Quinones R, Rosobin A, Trepel J, Sausville E, Phares J, Gress R, Poplack D, Weber J, Schechter G, Neckers L. The beta subunit of the IL-2 receptor mediates IL-2 induction of anti-CD3 redirected cytotoxic capability in large granular lymphocytes. Blood 1988;71:825.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08904-04 OD
FORMERLY:
Z01 CB 09015-03 LTIB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of Protein Kinases in Modulating Cell Growth and Malignant Transformation

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

PI:	Wayne B. Anderson	Research Chemist	OD DCBD NCI
Others:	Chang H. Ahn	Biotechnology Fellow	OD DCBD NCI
	Mary Hinzman	ERTA Fellow	OD DCBD NCI
	Zoltan Kiss	Visiting Scientist	OD DCBD NCI
	Won Chul Choi	Visiting Scientist	OD DCBD NCI
	Sung Ae Lee	Biotechnology Fellow	OD DCBD NCI
	Jennifer Strasburger	Technician	OD DCBD NCI

COOPERATING UNITS *(if any)*
David Klein, Lab. Develop, Neurobiol., NICHD, NIH
Rayudu Gopalakrishna, Dept. of Pathology, UCLA, Los Angeles, CA

LAB/BRANCH
Office of the Director

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.15	2.85	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.)*

Studies have been carried out to better elucidate the regulatory properties of protein kinase C (PK-C). Results of investigations with rat pinealocytes indicate that an elevation of Ca^{2+} influx alone can initiate activation of PK-C. Treatments which elevate Ca^{2+} influx, including increased extracellular K^+ and addition of the Ca^{2+} ionophore A23187, cause redistribution of PK-C to the membrane fraction. It was established that a close correlation exists between redistribution (activation) of PK-C by phenylephrine, K^+, and A23187 and their ability to potentiate ß-adrenergic stimulation of cAMP and cGMP accumulation. Exposure of PK-C to low concentrations of H_2O_2 resulted in the rapid and parallel loss of phosphotransferase activity and phorbol ester tumor promoter binding. This oxidative inactivation of PK-C also occurred in intact cells exposed to a low concentration of H_2O_2, and suggests that such a free radical inactivation may be operative within cell populations to decrease PK-C activity. Treatment of P388 murine leukemic cells with the antineoplastic drug diaziquone (AZQ) causes a rapid and pronounced inactivation of PK-C apparently through a mechanism involving the formation of free radicals. Protein kinase C activities have been determined in control (drug sensitive) and multidrug-resistant human breast cancer KC cells, human KB carcinoma cells, and murine leukemic P388 cells. In all cases the drug-resistant cells expressed higher activities of both cytosolic and membrane PK-C compared to control, drug-sensitive cells. These data suggest that an increase in a specific form of PK-C may play a role in the development and modulation of multidrug resistance.

effects of adriamycin. These data suggest that an increase in a specific form of PK-C may play a role in the development and modulation of multidrug resistance.

Publications:

Gopalakrishna R, Anderson WB. Susceptibility of protein kinase C to oxidative inactivation: Loss of both phosphotransferase activity and phorbol diester binding, FEBS LETT 1987;225:233-37.

Thomas TP, Talwar HS, Anderson WB. Phorbol ester-mediated association of protein kinase C to the nuclear fraction in NIH3T3 cells, Cancer Res 1988;48: 1910-19.

Ho AK, Thomas TP, Chik CL, Anderson WB, Klein DC. Protein kinase C: Subcellular redistribution by increased Ca^{2+} influx, J Biol Chem 1988; in press.

Thomas TP, Gopalakrishna R, Anderson WB. Assessment of hormone and tumor promoter-induced activation (membrane association) of protein kinase C in intact cells. In: Conn PM, Means AR, eds. Methods in Enzymology: Cellular Regulators. Orlando: Academic Press, 1987;399-411.

Major Findings:

Studies have been aimed at characterizing the biochemical events involved in modulating protein kinase C (PK-C) activity to regulate intracellular processes such as cell proliferation, tumor promotion, and resistance to drugs of the natural products class. A variety of hormones and growth factors interact with their cell surface receptors to stimulate phosphatidylinositol (PI) turnover and generate diacylglycerol, a physiological activator of PK-C. Previous studies have established that treatment of intact cells with such extracellular agents causes a rapid redistribution, or stabilization (activation) of PK-C to membranes.

Phenylephrine treatment of rat pinealocytes has been shown to stimulate PK-C redistribution to the particulate fraction. Results now establish that the receptor mediating this effect belongs to the alpha-adrenoceptor subclass. Reducing extracellular Ca^{2+} with EGTA or inhibiting Ca^{2+} influx with inorganic Ca^{2+} channel blockers decreases the phenylephrine-induced translocation of PK-C. Further, treatment of cells with agents known to increase Ca^{2+} influx into the cell (alpha-adrenergic agonists, depolarizing concentrations of K^{+} or the Ca^{2+} ionophore A23187) can initiate the rapid redistribution (activation) of PK-C at the plasma membrane. The effect of K^{+} was blocked by nifedipine and that of A23187 by EGTA, indicating that the effects of these agents are Ca^{2+}-dependent. These results indicate that an increase in Ca^{2+} influx in intact cells may be sufficient to trigger membrane stabilization (activation) of PK-C, and suggest that the transport of extracellular Ca^{2+} into the cell may be as critical to the hormonal modulation of PK-C as is the generation of the second messenger diacylglycerol.

Loss of both PK-C activity and phorbol ester binding occurs in cells treated for prolonged periods of time with phorbol ester. Results indicate that PK-C both in the isolated form and in intact cells is highly susceptible to oxidative inactivation upon exposure to hydroxyl radicals (OH). This oxidative inactivation results in the loss of both phosphotransferase activity and phorbol ester binding. Prior treatment of cells with TPA to induce membrane association of PK-C, followed by exposure to H_2O_2, results in increased inactivation of PK-C. This indicates that association of PK-C with membranes increases its susceptibility to oxidative inactivation. Also, treatment of P388 murine leukemic cells with the antineoplastic drug diaziquone (AZQ), which apparently acts through the generation of free radicals, also causes the rapid inactivation of PK-C.

Several lines of evidence indicate the possible involvement of PK-C in modulating cellular resistance to antitumor drugs of the natural products class. Both cytosolic and membrane PK-C activities are found to be markedly increased in drug-resistant compared to control, drug-sensitive human breast cancer KC cells, human KB carcinoma cells, and murine leukemic P388 cells. Western blot analysis with antisera to PK-C indicates that the elevated PK-C activity measured in adriamycin-resistant KC cells is due to an increase in native 80KDA PK-C. Results of cell growth and cytotoxicity studies in which PK-C activity has been altered with TPA and with the H-7 inhibitor indicate a close relationship between PK-C activity and cell sensitivity to the cytotoxic

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08905-07 OD
FORMERLY:
Z01 CB 08256-06 LTIB

PERIOD COVERED
October 1, 1987 to September 30, 1988

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)*

PI:	Wayne B. Anderson	Research Chemist	OD DCBD NCI
	Chang H. Ahn	Biotechnology Fellow	OD DCBD NCI
	Jennifer Strasburger	Technician	OD DCBD NCI

COOPERATING UNITS *(if any)*
Charis Liapi, Dev. Endocrinol. Br. NICHD, NIH
Daniele Evain-Brion, Lab. Physiopath. Dev., Paris, France
Gary Grotendorst, Dept. of Biol., Univ. So. Fla., Tampa, FL

LAB/BRANCH
Office of the Director

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	0.4

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.)*

An early event of retinoic acid (RA) action to promote differentiation of embryonal carcinoma (EC) stem cells is to sensitize the cells to cyclic AMP by elevating cytosolic and membrane-associated cyclic AMP-dependent protein kinase (cAMP-PK) activities. Studies now indicate that RA treatment of F9 stem cells causes a rapid (within 1 hr) and pronounced (~50%) decrease in the R_I and R_{II} regulatory subunits, and in cAMP-PK activity, at the nucleus. This decrease in nuclear cAMP-PK activity may be an early event of retinoid action to mediate eventual cellular differentiation. Studies also have addressed the mechanism(s) by which retinoids act to antagonize the effects of phorbol ester tumor promoter (TPA). Treatment of PYS cells with TPA causes a rapid (within 10 min) increase in cAMP-PK activity in the cytosol and a concomitant decrease in this activity associated with membranes. Addition of RA simultaneously with TPA completely blocks the TPA effect on cAMP-PK. Studies have shown that cAMP-PK as well as the levels of the R_I and R_{II} regulatory subunits, are decreased in fibroblasts derived from skin biopsy samples of psoriatic adult patients. Retinoic acid treatment of psoriatic fibroblasts in cell culture induces a pronounced increase (within 3-4 hrs) in the amount of the R_I and R_{II} subunits and in cAMP-PK activity to levels comparable to that found in normal human fibroblasts. This in vitro effect of retinoids on cAMP-PK in psoriatic fibroblasts may explain, at least in part, some of the in vivo therapeutic effects of retinoids.

Major Findings:

The intent of this project is to elucidate the role, and mechanism of action, of retinoids and hormones in mediating cell growth and differentiation. Since protein kinase activities have been implicated in the regulation of these biological events, studies have been ongoing to determine changes in cyclic AMP-dependent protein kinase (cAMP-PK) activity in response to retinoic acid (RA). Previously, it was established that retinoid-induced differentiation of teratocarcinoma (F9) cells is mediated by cyclic AMP. Treatment of F9 cells with RA now is found to induce a rapid (with 1 hr) dramatic (50%) decrease in the R_I and R_{II} cAMP binding regulatory subunits, and in cAMP-PK activity, at the nucleus. This initial decrease in nuclear cAMP-PK activity may be an early event of retinoid action to mediate eventual cellular differentiation. At later time periods (10 to 20 hr) after RA treatment of F9 cells there is an increase in both cytosolic and membrane-associated cAMP-PK activity. This later increase in cAMP-PK activity may regulate the direction (to visceral or parietal endoderm) of this differentiation process.

Retinoids have been shown to act as antagonists towards some of the effects of phorbol ester tumor promoter (TPA) in many cell systems. Treatment of PYS cells with TPA previously has been shown to enhance protein kinase C activity associated with membranes and to decrease activity of this enzyme in the cytosol. Evidence now indicates that TPA treatment of PYS cells causes an opposite effect on cAMP-PK activities. Within 10 min of treatment with TPA cAMP-PK activity shows an increase in the cytosol with a concomitant decrease in the membranes, as measured both by peptide phosphorylation activity and by photoaffinity labeling of R_I and R_{II} subunits with 8-azido-cyclic [^{32}p] AMP. Morevoer, the anti-tumor promoter RA, when added simultaneously with TPA, completely abolished the TPA effects observed on cAMP-PK. These results suggest that TPA induces a rapid redistribution of cAMP-PK between the membrane and cytosolic fractions. That RA blocks this TPA-induced translocation of cAMP-PK suggests that early antagonistic effects of RA toward TPA-mediated events occur at the plasma membrane.

Since retinoids have been used with limited success to treat the hyperproliferative skin disease psoriasis, studies were carried out to determine if exposure of human psoriatic fibroblasts with RA might sensitize these cells to cAMP. Previously, it was demonstrated that cAMP-PK activity is decreased in psoriatic cells, and that the amount of the R_{II} regulatory subunit is significantly decreased, or undetectable, in cytosol prepared from psoriatic cells. Results now indicate that RA treatment of psoriatic fibroblasts induces a pronounced increase in the levels of the R_I and R_{II} subunits, and in cAMP-PK activity. These results show that retinoid treatment of psoriatic cells overcomes the defective cAMP-PK system and helps to restore normal growth regulation mediated by cAMP.

Publications:

Raynaud F, Leduc C, Anderson WB, Evain-Brion D. Retinoid treatment of human psoriatic fibroblasts induces an increase in cyclic AMP-dependent protein kinase activity, J Invest Dermatol 1987;89:105-110.

Plet A, Evain-Brion D, Gerbaud P, Anderson WB. Retinoic acid-induced loss of nuclear cAMP-dependent protein kinase in F9 cells, Cancer Res 1987;47:5831-4.

Liapi C, Gerbaud P, Anderson WB, Evain-Brion D. Altered hormonal responses: Markers for embryonal and embryonic carcinoma stem cells and their differentiation derivatives, J Cell Physiol 1987;133:405-8.

Grotendorst GR, Harvey AK, Nagarajan L, Anderson WB, Gatewood E. Differentiation-dependent production of a platelet-derived growth factor-like mitroattractant by endoderm cells derived from embryonal carcinoma cells, J Cell Physiol 1988;134:437-44.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08212-14 OD

PERIOD COVERED

October 1, 1987 to September 30, 1988

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
	Richard E. Manrow	Senior Staff Fellow	OD DCBD NCI
	Marc S. Krug	Senior Staff Fellow	OD DCBD NCI
	Adriana R. Sburlati	Visiting Associate	OD DCBD NCI
	Alvaro Leone	Visiting Fellow	OD DCBD NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

OD, DCBD

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.3	4.3	

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 prothymosin α gene family consists of 7 members, 6 of which appear to be pseudogenes. The seventh gene is clearly functional and gives rise to two 1.3 kb mRNAs. Apparently, alternate splicing events in some tissues include a glutamic acid codon in the processed transcript resulting in a protein with one additional amino acid. The functional gene contains 5 exons, which have been sequenced; the 4 introns occur within the 333 bp coding region. Upon analyzing the 5'-ends of all members of the family, no signal peptide sequences were found. Six genes were very closely related with greater that 90% sequence homology at both the nucleic acid and amino acid levels. As a consequence of an upstream initiator codon and a 7 base pair deletion, the most divergent member of the group encodes a totally different protein. These data rule out the possibility the prothymosin α, regardless of which gene is expressed, is secreted. Therefore, based on our previous work, prothymosin α must be construed as a mature intracellular protein that plays a role in proliferation in a wide variety of cells. Earlier views claiming that prothymosin α was a precursor for thymus specific hormone-like peptides called thymosins are incorrect.

Prothymosin α is an inappropriately named protein which was thought to be a precursor for the thymus-specific peptide hormone called thymosin α_1. Thymosin α_1 was believed to restore, maintain or enhance immune function and to play a regulatory role in the differentiation of T lymphocytes. As a result of previous work in this laboratory, it became clear that these views were entirely incorrect. We found that prothymosin α mRNA is present in most, if not all, cells at levels dependent on their proliferative state, that prothymosin α is a mature intracellular protein rather than a precursor for peptides, and that it appears to participate in processes involved in the transition from cellular quiescence to active growth. Thymosin α_1, composed of the first 28 amino acids of prothymosin α, is an artifact.

Our present conception of the role of prothymosin α stems from the isolation and analysis of two cDNA clones, one each from a human fibroblast and human lymphocyte library. The encoded sequences of these clones are homologous but not identical. Both polypeptides are highly acidic, devoid of aromatic amino acids, and lacking in the amino-terminal hydrophobic signal peptide sequence required for translocation into the endoplasmic reticulum and eventual secretion from the cell. We have also established that a 1.3 kb prothymosin α mRNA is induced upon growth stimulation.

Experiments performed in the past year have confirmed and extended these conclusions by focusing on the isolation and characterization of genomic DNA clones for prothymosin α. If there were several genes for prothymosin α, the possibility remained that one or more genes encoded a secreted protein with some or all of the properties of thymosin α_1. Examination of genomic DNA from five individuals by means of Southern blots hybridized with prothymosin α cDNA probes revealed a complex pattern consistent with multiple genes and/or introns.

To resolve the issue, we screened 2 human cosmid libraries in search of prothymosin α sequences. We expected to find 4 genes because genomic digests of many human DNA samples with EcoRI resulted in 4 discrete fragments capable of hybridizing to both the 5'- and 3'-terminal sequences of the full-length cDNA. Instead, we found 6 cosmid clones each of which met the aforementioned criteria for a virtually complete gene. By restriction digestion analysis, we ascertained that the 6 cosmids represented 6 different genes. However, none of these isolates contained the smallest of the EcoRI bands (2-3kb) found in Southern blots of human DNA. We therefore constructed a plasmid genomic library with EcoRI digested human DNA enriched for the appropriate size class. This approach eliminated the possibility of reisolating clones identical with those already in our collection. After screening 250,000 out of a possible 10^6 clones at our disposal we found yet another gene, different from the rest. We believe we have now isolated all the genes for prothymosin α.

The 5'-ends of the 7 prothymosin α genes were sequenced in order to locate putative signal peptides. We found no genes that encoded a prothymosin α sequence with a signal peptide. We now feel that prothymosin α cannot be secreted by processes known to occur in living cells and that prothymosin α or fragments thereof, found in the serum of normal or diseased patients, reflect cell death.

Preliminary analysis of the genes disclosed one, called p3.28E, which most closely reflected the sequence of both cDNAs. This gene is approximately 4.7 kb in size and, by inspection, could be transcribed into the correct mRNA. It has 5 exons, identified in nuclease protection experiments as approximately 220, 70, 120, 70 and 730 bp in 5' to 3' order. We have sequenced all the exonic material and the exon-intron junctions and obtained the exact sizes of the exons. It is interesting to note that all the introns interrupt the 333 bp coding region of the gene. To date there are 7 single base differences with the full-length cDNA, all of which occur in noncoding regions. Because we have not found 2 closely related genes, one for each cDNA, we suspect that the cDNA clones are derived from alternate splicing of a single gene. The nucleotide sequence in the region involved is consistent with this view.

Nuclease protection experiments with genes that have several small exons of approximately the same size are difficult to interpret. Since the number of exons is one greater than the number of introns we devised an independent method for counting exons by measuring introns. The method is the inverse of the nuclease protection technique. It requires hybridization of gene and cDNA, treatment with RNase H to remove exons, fractionation of the surviving fragments electrophoretically, blotting, and hybridization of the blot with gene fragments. This technique not only counts introns but also yields their sizes and the order in which they appear in the gene. We found 4 introns in the prothymosin α gene which from 5' to 3' are approximately 2.6, 0.49, 0.49 and 0.30 kb, respectively. The sizes of the three smallest introns have been confirmed by sequencing.

The remaining 6 prothymosin α genes have not yet been thoroughly analyzed. It is clear, however, that one is a distant cousin; although the nucleic acid sequences are homologous, an upstream initiator codon and a 7 bp deletion result in an open reading frame that encodes an entirely different protein. None of the 6 genes has an intron in the position of intron 1 or 2 of p3.28E. Indeed, we are not sure that any of the genes, with the exception of p3.28E. has an intron. We also do not know whether the others are expressed.

The ribonuclease H activities associated with retroviral reverse transcriptases are believed to possess substrate specificities different from those of cellular ribonucleases. There is evidence that the RNase H activity of reverse transcriptase isolated from avian myeloblastosis virus functions as an exonuclease whereas normal cellular RNase H's are random endonucleases. With the advent of AIDS, it has become acutely important to rectify flaws in previous work in order to identify and to exploit differences between normal and viral-specific enzymes. Toward this aim, we have devised a perfect circular DNA:RNA hybrid which can be used to determine the substrate specificity of the AIDS reverse transcriptase-associated RNase H. The substrate can also be used to determine the type of termini preferred by the enzyme should it prove to be an exonuclease. Synthesis of this material was technically very demanding, requiring several innovative enzymatic approaches. We have now successfully completed construction of the substrate and we have devised and carried out tests of its quality. The substrate is correct. Using this material, we have tentatively characterized the RNase H activity of

reverse transcriptase from avian myeloblastosis virus and plan to characterize the HIV enzyme shortly. These results might contribute to a novel drug for AIDS.

Publications:

Berger SL, Chirgwin JM. Isolation of RNA. In: Dahlberg JE, Abelson J, eds. Methods Enzymol. San Diego: Academic Press, in press.

Berger SL. Nuclease digestion: A method for mapping introns. In: Dahlberg JE, Abelson J, eds. Methods Enzymol. San Diego: Academic Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08906-01 OD

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Synthetic HIV Protease Gene and Its Expression

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

P.I. John M. Louis	Staff Fellow	OD DCBD NCI
P.T. Mora	Supervisory Research Chemist	OD DCBD NCI
C.A.D. Smith	Visiting Associate	OD DCBD NCI

COOPERATING UNITS *(if any)*

S. Oroszlan, Bionetics, NCI, FCRF

LAB/BRANCH

OD, DCBD

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	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.)*

Nucleotide sequence of the gag-pol region of the HIV-1 virus, encoding for a 99 amino acid long protease was synthesized in the pol open reading frame. After additions of the translational initiation and termination codons to the appropriate ends, the protease gene was inserted into a high expression vector which was then used to transform E. coli cells. The recombinant clones bearing the correct coding sequence of 297bp were analyzed for expression of the protease gene product using specific antibodies. The product was detected in Western blots as a single 11.5kd protein. After selecting the optimum conditions for expression of this gene and solubilization of the protein, the specific proteolytic activity was proven by its capacity to cleave correctly a synthetic nonapeptide, corresponding to the HIV-1 p17-p24 cleavage site. This cleavage, together with others, is necessary for the processing of the gag-pol precursor, and thus to the retroviral life cycle. Specific inhibitors of the retroviral protease, now being sought, might be effective anti-retroviral therapeutic agents.

GPO 914-918

Major Findings:

Synthesis of the Full Length Protease Gene. The nucleotide sequence of the protease gene and its complement were synthesized as five individual fragments of approximately 60 bases. Translational initiation codon ATG and termination codon TAA were provided to the appropriate ends of the protease gene. Compatible restriction endonuclease recognition sequences were also added and ligated to the linearized expression vector and the resulting construct was used to transform E. coli cells for expression.

Expression of the Synthetic HIV-1 Protease Gene in E. Coli. Three clones bearing the correct coding sequence of 297bp in the expression vector PKK233-2 were analyzed for expression and to select conditions for the optimal induction of the gene by Western blot analysis. The cloned gene expressed a single, unfused protein of 11.5kd. Different buffer systems were used for the lysis of cells after optimal induction. Sonication in a buffer system of 50mM Tris-Hcl at pH 7.5, 1mM DTT, 1mM PMSF and 0.5% nonidet P-40 released 50% to 70% of the protease in the soluble fraction.

Demonstration of Specific Proteolytic Activity. To assess the activity of the cloned HIV-1 protease a synthetic nonapeptide corresponding to the HIV-1 p17-p24 cleavage site was used as a substrate. Equal aliquots of incubation mixture were taken at various time points and analyzed by RP-HPLC. The substrate in the 0 hour sample eluted as a single peak. After incubation for 30 min., two newly appearing peaks, labelled product 1 and product 2, can be seen correlating with a significant decrease of the substrate peak. Subsequent amino acid analysis of the recovered peaks demonstrated that product 1 and product 2 corresponded to the expected cleavage products. No cleavage products have been detected in reactions using extracts from uninduced cells and of control cells and there was no decrease in the substrate peak even after 48 hours of incubation indicating that the nonapeptide is resistent to degradation by bacterial proteases. This makes this substrate especially useful for assaying protease activities in crude extracts. The enzyme has been partially purified by specific column chromatographic techniques, including affinity chromatography.

The objective of the ongoing experiments is to produce enough active protease to study the structure of the protease and its mechanism of action, with a goal of devising specific inhibitors to this enzyme which is essential to the life cycle of the virus.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05526-20 OD

PERIOD COVERED
October 1, 1987 to September 30, 1988

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
	C.A. Dale Smith	Visiting Associate	OD DCBD NCI
	John M. Louis	Staff Fellow	OD DCBD NCI
	V.W. McFarland	Chemist	OD DCBD NCI
	T. Keler	Guest Worker	OD DCBD NCI

COOPERATING UNITS *(if any)*
Frank Hetrick, University of Maryland; Anton Jetten, National Institute of Environmental Health Sciences; David Winterbourne, St. George's Hospital, University of London, Jean-Jacques Lawrance, INSERM, Grenoble, France.

LAB/BRANCH
OD, DCBD

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

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.)*

Expression of the phosphoprotein p53 and also several of the protooncogenes were studied in various differentiating systems. In the embryonal differentiation of the chicken the decrease in p53 protein was found to be caused by the decrease in the steady state level of the mRNA regulated by a post-transcriptional mechanism. In the differentiation of rabbit epithelial cells induced by added TGFβ or retinoic acid, or alternately by deprivation of EGF, very large changes in p53 mRNA and also in c-fos and H-ras mRNA were observed, with concomitant well defined changes in cell phenotypes resulting in secretory or in keratinoid cells. Studies on induced embryonal differentiation of carcinoma cells further supported the finding that a decline in p53 mRNA (through a post-transcriptional mechanism) is a general correlate in cellular differentiation. This finding is important, as together with the finding of the conservation of the p53 gene during evolution of the vertebrates from fish through avian to mammalian species, suggests an essential function for p53.

p53 cDNA clones were obtained from 3T12 Balb/c mouse cells in which the p53 is unable to complex with the SV40 T antigen. Sequencing and transfection assays are now being carried out to assess if naturally occurring structural changes in the p53 (detectable also by specific monoclonal antibodies) cause the inability for complexing.

Major Findings:

1. Evolutionary Conservation of the p53 Gene and Other Oncogenes.

We have screened established fish cell lines derived from catfish epidermis, carp epidermis, rainbow trout gonad and Chinook salmon embryos for the presence and expression of genes related to the mammalian and avian oncogenes. We have derived sequences homologous to p53, ras, myc, raf, erb-b, src, abl, fos, and sis, and are presently attempting to derive these genes from a gt-11 genomic library derived from DNA isolated from a "Brown-Bullhead" catfish cell line. The "Brown-Bullhead" catfish was chosen because of the temperature-dependent appearance of skin lesions and tumors on this species during the warm weather season in the inland waterways of Pennsylvania. (In collaboration with F. Hetrick.)

1. Complexing of the SV40 Large T ag. with p53.

a) Protein analysis: Screening a large number of different cell lines with several anti-p53 monoclonals and an anti-T monoclonal indicates that a significant number of SV40 transformed cells exhibit a marked reduction or complete lack of complex formation. Using an anti-p53 monoclonal, pab246, that recognizes a conformationally-dependent epitope on the protein, a direct correlation can be seen between the lack of (or reduced) recognition by this antibody before SV40 transformation and the extent of T ag.\p53 complexing after infection of cells with wild type SV40 virus. Cell lines that possess p53 protein that is virtually all able to react with pab246, show high levels of complexed p53 and T ag. on transformation by SV40. In many cell lines in which the p53-246 epitope is not detectable or low, a cellular protein of approximately 68kd. is co-immunoprecipitated with p53 protein using other monoclonals (e.g., pab421), but not with any of the p53 protein recognized by pab246. Half-life data suggest that p53 in the non-complexing SV40 transformed-cell types is not stabilized by the interaction with the cellular p68 protein, and the steady state level of p53 is governed by p53-mRNA. In 3T12 cells and derivatives, the p68 binding to p53 is distinct, however it does not respond to heat shock treatment.

It is evident that several distinct forms of p53 exist in most cells and the overall distribution and the form which predominates governs the extent to which T ag. can complex p53.

b) cDNA cloning of p53 from non-complexing mouse cells: cDNA clone of p53 obtained from balb 3T12 cells appear to show several nucleotide alterations from the normal sequence that may explain lack of 246 recognition, p68 binding and the absence of T ag. complexes after SV40 transformation.

2. Differentiation and Expression of Oncogenes.

a) P53 in chicken embryogenesis: During the embryonal development of the mouse and of the chicken, there is a decline in the steady-state levels of the p53 protein and an equal decline of p53 mRNA as measured by Western and Northern blots. During the development of the chicken, the relative rates of p53 transcription is constant as measured by nuclear run-on analysis. p53 mRNA is relatively stable (half-life >12 h) in both chicken and mouse embryos. We

concluded that (i) the down-modulation of p53 mRNA (and of protein) during embryonal development has been well conserved during the evolution of the vertebrate (this implies the p53 protein may have a function in embryonal development); and (ii) the mechanism is on a post-transcriptional level. Differences in the processing of the mRNA (maturation, transport, degradation) are being further studied in collaboration with J.J. Lawrance.

b) Induced differentiation in cell culture: Similar to the natural embryonal development, in induced differentiation of certain cell types which can be attained in a controlled fashion in tissue culture, a significant (2-5X) decrease in p53 mRNA and also of C-fos and Ha-Ras mRNA was observed. These changes occurred when induced differentiation is attained by crowded growth of rabbit tracheal epithelial cells, or by depriving these cells of EGF, or by treating them with TGF, which all will lead to the expression of squamous cell phenotype. When the same cells are treated with retinoic acid the cells express secretory phenotype, and in this case there is a concomitant increase (5X) in the level of C-fos mRNA. (Collaborative study with A. Jetten).

Another system studied was when changes occur in the phenotype of rat parotid acinar cells (glands) upon stimulation of the adrenergic-β receptor with the β-agonist isoprofeneral. In this system there was a very high (20-30X) and rapid (<60 min) increase in p53, C-fos and c-myc mRNA, which all can be prevented by the antagonist propranalol.

4. Affect of a Bacterial Preparation of Cell Growth in Culture.

a) Using a well defined family of cloned cells derived from a 10day balb-c mouse embryo, experiments on normal and SV40 transformed cells have shown that a purified lipid compound called DCX, isolated from endotoxin preparations of Serratia marcescens, displays a selective cytotoxic affect on the SV40 infected derivative cell line, having no affect on the untransformed counterpart. The structure of this compound is under investigation at the collaborator's laboratory. The cytotoxic affect of DCX on SV40 transformed cells occurs at doses at 2ng/ml. The cytotoxic affects of DCX is manifested in arrested growth, in inhibition of DNA synthesis, increased K^+ uptake, increased glucose uptake, decreased ATP production and in characteristic changes in cell morphology. The morphological changes occur only on the SV40 transformed cell. DCX appears to elicit this selective effect on cell morphology through cell membrane perturbation (observed through scanning electron microscopy), and through altered energy pathways (sugar utilization). (Work of C.A.D. Smith with T. Keler.)

Publications:

Smith CAD, Winterbourne DJ, McFarland VW, Mora PT. Changes in heparan sulphate pattern but not in oncogene expression correlate with tumor growth in spontaneous transformation of cells, Oncogene Res 1987;1:325-41.

Smith CAD, Louis JM, Hetrick FM. A sequence homologous to the mammalian oncogene, p53 in fish cell lines, J Fish Diseases 1988; in press.

Louis JM, McFarland VW, May P, Mora PT. The phosphoprotein p53 is down-regulated post-transcriptionally during embryogenesis in vertebrates, Biochim Biophys Acta 1988; in press.

Kousvelari E, Louis JM, Thang L, Curran T. Regulation of proto-oncogenes in rat parotid acinar cells in vitro after stimulation of β-andrenergic receptors, Exp Cell Res 1988; in press.

Nowotny A, Keler T, Smith CAD. Chemistry and biology of DCX, a novel lipid contaminant of some endotoxin preparations with selective cytotoxicity to transformed cells, Endotoxins 1988; in press.

Mora PT, Winterbourne DJ, Smith CAD. A clonal analysis of malignancy, Bioscience Rep 1988; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08903-02 OD

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

The Structure of Thyroid Hormone Precursors

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

Sidney Shifrin Chemist OD DCBD NCI

COOPERATING UNITS *(if any)*

Evelyn F. Grollman LMB NIADDK
Sonia Quateli, Clinical Hospital, Sao Paulo, Brazil
Richard Montali, National Zoological Park, Washington, D.C.
Leonard D. Kohn LMB NIAMSD

LAB/BRANCH

OD, DCBD

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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.)*

Dissociation of thyroglobulin to a 26,000-dalton protein by succinylation is not blocked by pretreating the glycoprotein with trinitrobenzenesulfonic acid (TNBS). Treatment of normal, human thyroglobulin with TNBS alone causes dissociation of the large glycoprotein into smaller peptides some of which have molecular weights as low as 10,000. 19S thyroglobulin prepared from a human endemic goiter is not dissociated by trinitrophenylation. Thus, the lysyl residues of thyroglobulin can be divided into two parts: the lysyl residues of 19S residual thyroglobulin and of the 10,000-dalton peptide react readily with TNBS but do not react with succinic analyaride. However, the lysyl residues of the 26,000 dalton peptide react readily with succinic anhydride and react poorly with TNBS.

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

Major Findings:

Heretofore it was believed that thyroglobulin (660,000 daltons) was composed of two identical subunits each having a molecular weight of 330,000 and held together by disulfide bonds. The first indication that the high molecular weight thyroglobulin was, in fact, composed of lower molecular weight peptides which are held together by non-covalent bonds was shown by me in collaboration with Dr. Annalisa Tanini from Firenze, Italy, in a paper which was published in the Journal of Biological Chemistry in 1983. We found that a lower molecular weight peptide (26,000 daltons) dissociated from 19S thyroglobulin following succinylation. Many oligomeric proteins dissociate into their constituent subunits following succinylation. The mechanism by which this happens is believed to be due to the introduction of the negatively charged succinate group which causes the subunits to dissociate as a result of electrostatic repulsions. That is, upon the succinylation of a few lysyl residues, the conformation of the protein is modified to such an extent that the intersubunit bonds are weakened. The introduction of more negatively charged succinate groups weakens the intersubunit forces even more until a subunit dissociates.

Since the publication in 1983, I, together with members of Dr. Leonard Kohn's laboratory, have found the 26,000-dalton subunit to be one of the major products synthesized by functional rat thyroid cells in tissue culture. The major effort of our investigation is now concerned with examining the reactions of the ε-amino groups of lysine residues of 19S human thyroglobulin isolated from the thyroid glands of patients without a history of thyroid disease and from patients with endemic goiters, (from Brazil) with Graves' disease and with Hashimoto's disease. These studies have included work on animal thyroids generously provided by Dr. Montali.

Reaction of thyroglobulin with reagents which react selectively with the ε-amino group of lysyl residues but do not introduce a full positive charge also causes the 26,000-dalton peptide to dissociate. One of these reagents is 2,4,6-trinitrobenzenesulfonic acid (TNBS) which introduces a trinitrophenyl (TNP) group into the molecule. When we react 19S normal human thyroglobulin with TNBS, a peptide is released which is poorly substituted by the TNP-group. However, the residual 19S TNP-thyroglobulin is highly substituted. A manuscript summarizing our findings has just been completed.

Preliminary studies have just been undertaken with the assistance of a summer student, Jocelyn Joseph, from Xavier College. From studies that I carried out many years ago I recalled that the aldehyde group of pyridoxal phosphate (Vitamin B_6) can form a Schiff base with the ε-amino group of lysine. When Ms. Joseph and I added pyridoxal phosphate to thyroglobulin, we observed that only the lysyl residues of the 26,000-dalton peptide were modified with this coenzyme. We are presently studying the possibility of reversing this reaction so that we can examine the physicochemical properties of the unmodified 26K-dalton peptide.

These observations introduce many questions into the structure of thyroglobulin, in particular, and into the structure of oligomeric proteins in general. For example, what is the nature of the forces which hold the 26K

peptide to the much larger thyroglobulin molecule? Are these forces involved in the delivery of the thyroid hormones T_3 and T_4 to the animal?

Relevance to the Mission of NCI. Very little is known about the structure of thyroglobulin extracted from patients with thyroid tumors. My studies are directed at a complete elucidation of the structure of thyroglobulin in normal patients and in patients with a thyroid tumor. I already know that thyroglobulin extracted from patients with a tumor or from the Bongo do not dissociate after trinitrophenylation. Why doesn't it dissociate?

Future Studies. I would like to get some thyroid tumors which have been surgically removed from patients (if possible) so that I can further characterize this large glycoprotein in diseased tissue. My studies on chemical modifications are continuing with collaborations with Dr. Alan Minton and Dr. William Coleman. Dr. Minton has devised sensitive methods of determining the molecular weight of proteins and Dr. Coleman has devised methods of determining the sugar composition of the oligosaccharide portion of glycoproteins.

Methods Employed. Isolation of thyroglobulin from humans and from animals, protein purification, chemical methods of derivatization, radiolabelling, radioimmunoassay for T_3 and T_4, sedimentation velocity ultracentrifugation, sedimentation equilibrium ultracentrifugation, absorption spectroscopy, fluorescence spectroscopy, structure determination of the oligosaccharide moiety of thyroglobulin and its subunits.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00941-32 OD

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Genetic and Other Factors Affecting Marrow Transplantation on Irradiated Mice

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

PI: Delta S. Uphoff Research Biologist OD DCBD NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

OD, DCBD

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)*

Successful marrow transplantation requires not only genetically compatible donors but the elimination of normal or neoplastic hematopoietic cells of the recipient without destruction of other vital tissues. Among the factors influencing the effectiveness of radiation treatment of the hematopoietic system was exposure rate and direction of the exposure. Simultaneous two-directional exposures were most effective. Exposure rate reduced the effectiveness of dorsal exposures while exposure rate plus lack of homogeneity further reduced the effectiveness of ventral exposures. Continuous reciprocal alternate exposures were used to establish the time required to initiate repair when a reduced exposure rate was used. Compliance with the requirement of the Animal Welfare Act that sedation be used to eliminate stress during treatment may reduce the biological effectiveness of the radiation treatment and the success of the bone marrow transplantation. Other physical factors may also affect the success of bone marrow transplantation but have proved to be less readily investigated.

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

Initially attention was focused primarily on the selection of compatible donors for marrow transplantation. The ability to select compatible organ donors has resulted in predictable success for transplantation of many tissues. The notable exception has been marrow transplantation. Although the hematopoietic system is radiation sensitive, successful depletion of this system in preparation for a marrow graft requires greater precision than was realized heretofore. Treatment is complicated by the diverse nature of the system which includes circulating cells, fixed cells of the spleen, lymph nodes and thymus and those encased in skeletal bone. While this diversity may have survival advantage in cases of radiation accidents, it complicates the treatment of neoplasms where survival of a few neoplastic cells may be the difference between success and failure of the treatment. In addition there has been no general agreement on the method of treatment. On the assumption that hematopoietic cells have very little repair capacity, very low exposure rates and many small fractions have been used.

Since all radiation produces cellular damage the aim should be to achieve the greatest biological effects from the lowest possible exposure. This was achieved by using simultaneous two-directional (dual) exposures. It has long been assumed that this beneficial effect resulted from the greater homogeneity of the dual exposure. This phenomenon has now conclusively been demonstrated to be an exposure rate effect rather than a result of increased homogeneity, i.e., dual and dorsal exposures at the same exposure rate produced identical results. Homogeneity of the exposure became a significant factor only when a significant portion of the target tissue was distal to the radiation source as in the case of ventral exposures compared with dorsal exposures. Contrary to common belief hematopoietic cells have a significant repair capability. Thus, attempts to increase homogeneity by continuous alternate exposures not only failed to increase the effectiveness of the treatment but were less effective than the unidirectional dorsal exposure. Reciprocal alternate exposures were not equally effective and demonstrated that repair was initiated only during exposure. Since the direction of the first exposure established the effectiveness of the treatment, initiation of repair required longer than 5 minutes to become established but significant repair was apparent after exposures of 11 minutes. Theoretically the effectiveness of the treatment might improve if multiple exposures of less than 5 minutes were employed. Preliminary data indicate that these observations are not unique for x-ray but also apply to gamma radiation from cesium 137.

There are numerous other factors which affect the effectiveness of radiation treatment. For example, compliance with the requirement of the Animal Welfare Act that experimental animals be sedated to reduce stress during treatment may have an adverse affect on some treatments. Some sedatives have a radioprotective effect. Sodium pentobarbitol has an immediate radioprotective effect which varies in degree with time. This effect is independent of the degree of sedation and has a rebound effect after apparent recovery. Sedation prior to or during irradiation should be tested for any possible residual effects.

Publications:

Uphoff DE, Swain RW, Bowie GM. Directional effects of acute total-body x-irradiation: Exposure rate versus homogeneity of exposure. JNCI 1988, in press.

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-85607
Starting Date: 11/1/87 Expiration Date: 10/31/93

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: This contract represents predominantly a facility for holding experimental animals in support of the research of the Immunology Branch. As such, the contractor has maintained a colony of 12,000 mice, 500 rats, and 40 rabbits. Breedings of certain strains of mice and rats for experimental needs of the Branch have also been performed when such animals have not been available commercially. Frozen samples of sera and cells are stored in freezers of appropriate temperatures and are transferred to NIH as required.

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 Officers: Dr. David H. Sachs and Dr. John R. Wunderlich
Program: Immunology Resource
Technical Review Group: Intramural Support Contract Subcommittee A
FY 88 Funds: $728,572 B

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-51014
Starting Date: 9/30/85 Expiration Date: 9/29/88

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, preparation of radiation chimeric mice, thymus transplants and embryo transfers. Protocols and details of experiments are to be carried as directed by the Project Officer.

Progress: Performance of this contract has been satisfactory. A number of experiments involving virus infection, allogeneic lymphocyte transfer, and combinations of the above have been performed in several mouse strains to investigate the synergistic effects of cytomegalovirus and graft-vs-host reaction. Approximately 6200 mice were received into the contract facility during the year, and the facility contained between 2500 and 2800 mice at any given time. One preparation each of cytomegalovirus and influenza virus were made. Approximately 600 radiation chimeras, 3200 i.p. inoculations, 1200 i.v. injections, 500 tail bleeds, 320 thymus grafts, 30 virus plaque assays, 150 skin grafts, 2600 embryos were transplanted into psuedopregnant mice, 80 lymphocyte preparations, 600 tumor palpations, 80 cytotoxic T lymphocyte assays, preparation of 60 ml of ascites fluid, and 800 ELISA tests for cytomegalovirus antibodies were performed during FY 1987. The mice have been delivered to the Immunology Branch laboratories as requested, and record keeping of stock mice and experimental protocols had been accurate.

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 Resource
Technical Review Group: Intramural Support Contract Subcommittee A
FY 88 Funds: $29,634 B

Contract Research Summary

Title: Characterization of HLA Antigens of Donors' Lymphocytes

Principal Investigator:	Dr. David Eckels
Performing Organization:	Blood Center of Southeastern Wisconsin
City and State:	Milwaukee, WI
Contract Number:	NO1-CB-33935
Starting Date: 7/1/83	Expiration Date: 6/30/88

Goal: To analyze as carefully as possible the cell surface histocompatibility antigens on donors' cells in order to subsequently analyze the relationship between those antigens and the ability of those donors' cells to mount appropriate immune responses.

Approach: Because of budgetary restrictions, the analysis of cell surface phenotype is currently restricted to analysis of the HLA-DP molecules by cellular proliferation in a PLT assay. That PLT assay involves analyzing secondary restimulation of lymphocyte populations selectively immunized against alloantigens in mixed lymphocyte culture.

Progress: During the last year the contractor has finished analysis of DP-typing of the unique 111 cell panel of lymphoblastoid B cell lines which was assembled under the aegis of the 10th International Histocompatibility Workshop and presented that data at the Workshop in the fall of 1987. The contractor has also DP-typed cells from panels of patients for analysis of HLA regulation of disease, particularly patients with multiple sclerosis and with dermatitis herpetiformis. In addition, typing has been on done on cells from panels of normal donors whose cells are being used for in vitro immunogenetic analysis.

The contractor has supplied information and reagents to several laboratories in the United States and around the world to enable them to develop their own expertise in HLA-DP typing. The contractor has submitted in a timely fashion the results of the typing done. The data from the typing are informative; the results for control cells indicate that the assays have been technically excellent.

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 mechanisms and genetic control.

Project Office	Dr. Stephen Shaw
Program:	Immunology Resource
Technical Review Group:	Ad Hoc Intramural Technical Review Group
FY 88 Funds: None	

A

CONTRACT RESEARCH SUMMARY

Title: Radioimmunoassay and Enzyme Immunoassay of Immunoglobulin Molecules and Antibodies

Principal Investigator: Norman Beaudry
Performing Organization: Hazleton Biotechnologies Corp.
Vienna, Virginia

Contract Number: NO1-CB-71010
Starting Date: 6/30/87 Expiration Date: 6/29/93

Goal: To perform radioimmunoassays of immunoglobulin molecules as well as ELISA assays of soluble interleukin-2 receptor molecules in lymphocyte culture supernatants or in biological fluids.

Approach: The contractor is to quantitate human immunoglobulins in various fluids using double antibody radioimmunoassay procedures and reagents defined and supplied by the project officer. In addition the contractor is to utilize an established ELISA assay for the soluble form of the IL-2 receptor to quantitate the level of this peptide in the serum of patients. 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 primary and acquired immunodeficiencies including those associated with a high incidence of malignant transformation and with human B- and T-cell leukemias.

Progress: The contractor has established the required radioimmunoassays for immunoglobulin molecules and the ELISA assays for the soluble form of the Tac peptide of the IL-2 receptor. Elevated receptor levels have been demonstrated in the sera of patients with HTLV-I Adult T cell Leukemia, HIV Associated AIDS, Hairy Cell B Cell Leukemia, or Hodgkin's Disease. Effective therapy of these leukemias and lymphomas was associated with a normalization of the serum IL-2 receptor levels.

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 regulation of humoral immunity. They are required for therapeutic protocols involving the use of the anti-Tac monoclonal antibody. The studies of circulating IL-2R peptide levels are of importance in defining the biology of neoplasia, as an aid in diagnosis and in monitoring therapy of IL-2 receptor positive malignancies.

Project Officer: Thomas A. Waldmann, M.D.
Program: Cancer Biology Resource
Technical Review Group: Ad Hoc Technical Review Group
FY: 1988 Funds: $226,059

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: Hazleton Laboratories
City and State: Rockville, MD

Contract Number: NO1-CB-71085
Starting Date: 02-01-87 Expiration Date 01-31-92

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: Contractor has carried out basic plasmacytoma induction experiments using pristane alone in various BALB/c.DBA/2 congenic C x S RI, (BALB/c x DBA/2)F_1 and BALB/cAn.BALB/cJ congenic strains. In addition, contractor has carried out plasmacytoma induction experiments in pristane conditioned mice with RIM, IM, R, J_2, J_3, J_5 and other myc containing viruses. Contractor transplants essential tumors and supplies tissues and/or DNA to investigators in the Laboratory of Genetics for molecular studies. Contractor ships mice, tumors, DNA, serum or ascites to other investigators. Contractor continues to develop essential congenic strains that are being used to identify new genes.

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, Dr. Beverly Mock
Program: Immunology Support
Technical Review Group: Intramural Support Contract Proposal Review Committee
FY 88 Funds: $742,212 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: Hazleton Laboratories America, Inc.
City and State: Rockville, MD

Contract Number: NO1-CB-71091
Starting Date: 2/1/87 Expiration Date: 1/31/93

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 maintained a colony of approximately 40 special inbred and congenic resistant strains of mice by pedigreed brother-sister mating. Quality control testing was carried out at each generation by cytotoxicity typing of breeders from each strain. Alloantisera were raised between mouse strains to assist in this quality control typing, and sera and animals were shipped by the contractor to collaborating investigators at NIH and elsewhere.

Progress: The contractor 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 were all highly satisfactory. A backcrossing program was used for all congenic resistant strains in order to keep the backgrounds of these strains identical. This involved backcrossing of each congenic to the reference background line once every 6-10 generations. This program has also been very satisfactory to date. Fourteen 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 were performed by the contractor, with the purpose of developing monoclonal antibodies to histocompatibility antigens. Antisera for histocompatibility antigen typing were prepared in various combinations and were found to be excellent reagents. A series of strain-restricted typing sera were 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 was satisfactory. The animals shipped were generally of excellent health and 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 were produced, stored and shipped starting with cell lines developed by the Project Officer.

Significance to Cancer Research: This animal facility was needed for the breeding and maintenance of these inbred congenic resistant strains of mice. These animals made 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 Resource
Technical Review Group: Intramural Support Contract Proposal Review Committee
FY 88 Funds: $655,529 B

CONTRACT RESEARCH SUMMARY

Title: Maintenance of a Feral Mouse Breeding Colony

Principal Investigator: Ms. Martha McGowan

Performing Organization: Hazelton Laboratories America, Inc.
City and State: Rockville, Maryland

Contract Number: NO1-CB-61000
Starting Date: 12-01-85 Expiration Date: 11-30-88

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 (cogenic) strains of feral M. musculus that contain specific genetically 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 germline-negative M. musculus CZECHII strain. Develop sublines of BALB/c and CZECHII V^- mice that have been foster nursed on high tumor incidence mouse strains. 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. Approximately 30 or more mammary tumors were obtained from each of the BALB/cfC3H, CZECHIIV$^-$fC3H, C3H, GR, CZECHII mouse strains. Other sublines of BALB/c mice foster-nursed on M. spretus, M. cervicolor propaeus tak, and CZECHII V^+ have been developed. MTV-1 and MTV-2 have been introduced into the CzechII V^- genetic background and are at backcross N6 and N8, respectively. Twelve mammary hyperplastic outgrowth lines from CZECHII V^+ mice are being carried to determine the frequency of mammary tumor development. Single cell suspensions of primary mammary epithelium have been introduced into cleared mammary gland fat pads and shown to repopulate the gland.

Significance to Cancer Research: Provides essential support for the study of mammary tumorigenesis with the specific goal of identifying and characterizing the genes at risk to MMTV activation. Provides essential biological material for other investigators studying the biology of the mouse mammary tumor virus as well as other classes of retroviral genomes.

Project Officer: Dr. Robert Callahan
Program: Immunology Resource
Technical Review Group: DEA; Ad Hoch Intramural Technical Review Group

FY 88 Funds: $124,893

*U.S. GOVERNMENT PRINTING OFFICE:1988-201-778:80351